

# Endogenous indole-3-acetic acid concentrations during adventitious root formation in Eucalyptus globulus

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# ENDOGENOUS INDOLE-3-ACETIC ACID CONCENTRATIONS DURING ADVENTITIOUS ROOT FORMATION IN *EUCALYPTUS GLOBULUS.*

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**for the degree of Ph.D.**
**of the University of Bath**
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# Contents.

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## Abstract.

A series of experiments were carried out to investigate rooting behaviour in *Eucalyptus globulus* seedling cuttings. Particular emphasis was placed on the effect of ageing, anatomical studies and on the quantification of endogenous IAA at key stages during adventitious root formation (ARF). The technique of GC-MS-SIM, in electron impact and chemical ionisation modes, was used for quantification of IAA, employing $^{13}C_6$-IAA as the internal standard. Anatomical studies showed that nuclear swelling, which occurred around thirty hours after cutting excision in seedling cuttings, was the first visible sign in ARF and was proceeded by early cell division and root primordium formation.

In the rooting zone of 'easy-to-root' *E. globulus* seedling cuttings there was a transient rise in the endogenous IAA level associated with the inductive stage of ARF, which subsided before the first cell divisions. IAA levels in the non-rooting zone of the cuttings remained fairly constant throughout the rooting process. The results were reproducible and a second experiment was carried out which identified the timing of events more precisely. In the second experiment similar changes in the levels of endogenous IAA were detected and the transient rise in IAA levels in the rooting zone subsided prior to nuclear swelling. Similar results were found with clonal *in vitro E. globulus* explants. The work in this thesis supports the hypothesis that IAA is associated with dedifferentiation and the formation of a new meristematic locus.

# Chapter 1. Introduction.

## 1.1 The genus *Eucalyptus*.

*Eucalyptus* species have a natural distribution predominantly confined to Australia, with limited occurrence of species in the islands to the north including Timor, New Guinea, Java and the Philippines. Large areas in South America, Africa, Asia, Spain, Portugal, Middle Eastern countries and the USA have been planted with eucalypts (FAO, 1979; Gupta and Mascarenhas, 1987). A major reason for the spread of eucalypts is the number of commercial uses they have. *Eucalyptus. delegatensis, E. regnans, E. saligna, and E. grandis* are the favoured trees for paper pulp production in Australia, whilst in Brazil and the Congo high quality pulpwood comes from *E. grandis, E. urophylla* and several hybrids (Mc. Comb and Bennett 1986). Essential oils from eucalypts are used for medicinal and perfumery purposes, and are being considered as components of liquid fuel (Calvin 1980; Stewart *et al.* 1982). The major producers of eucalypt oils are Portugal and Spain (*E. globulus*), Argentina, Chile, Brazil and Indonesia (*E. citriodora*), and Swaziland and South Africa (*E. dives*) (Donald 1980, Mc. Comb and Bennett 1986).

## 1.2 Clonal forestry.

Asexual propagation is an important commercial method available to the horticultural and forestry industry for producing large numbers of genetically uniform plants (Davies *et al.* 1982; Haissig *et al.* 1992). Cloning is also a critical component of many genetic improvement programmes for higher plants including those using conventional breeding and/or biotechnology techniques (Haissig *et al.* 1987). In addition to their long life cycle (Bonga

1982a), trees present a major problem in that many are highly heterozygous (Bonga 1982a; Franclet *et al.* 1987). This limits the use of inbreeding and controlled hybridisation as a means of obtaining genetic gain, for example due to various undesirable consequences of inbreeding depression (Libby *et al.* 1969; Winton *et al.* 1974). As a rule, eucalypts are considered to be out crossing (Goncalves *et al.* 1979). In theory, vegetative propagation should enable clonal plantations of superior trees that produce greater yields than seedling plantations (Zobel & Ikemori 1983). Productivity increase with sexual propagation requires several generations, whereas with vegetative propagation it is immediate (Bonga 1982a; Bennett *et al.* 1986; Franclet *et al.* 1987). When establishing plantations of dioecious trees, cloning can be desirable, as in some species only the female trees have economic value (Bonga 1982 b.; Jordan *et al.* 1983; Reynolds 1982). The potential for forest tree improvement via sexual breeding techniques is limited (Shelbourne 1987) and it is essential to develop vegetative propagation techniques. A major problem for many tree improvement programmes is that by the time trees are mature enough to identify elite genotypes, it is often impossible to propagate clonal material from them.

## 1.3 Maturation.

From the literature it is clear that maturation is a highly complex phenomenon. Much confusion arises from the different terminology which has been used to describe the process. Maturation can be defined as the developmental process inducing changes in morphological and physiological characteristics leading to the reproductive (mature) state (Hackett 1987). The

old terminology for maturation such as phase change, ontogenetic ageing and meristematic ageing will be avoided here. However, the use of the term meristematic ageing emphasises that meristems play an important role in maturation (Pierik 1987). One of the major factors underlying the process is increasing shoot apex size (Romberger 1976).

Associated with the transition from the juvenile to mature state are progressive changes in morphological and developmental attributes including leaf cuticular characteristics, bark characteristics, leaf shape, thickness, phyllotaxis, shoot growth vigour, rooting ability and stem pigmentation (Hackett1987). Changes in such characteristics during development vary from species to species and most changes occur gradually during the period preceding the mature phase (Hackett 1987). Even though the change in woody plants has been described frequently, systematic attempts to carefully document the time course of maturation events are lacking (Greenwood 1987). Maturation is highly significant both theoretically and practically. It is of importance to plant propagators in three main areas, the rooting of cuttings, the subsequent field performance of the cuttings, and in woody plant breeding (Clark 1982)

In a small number of woody plant genera, juvenile tissue is the only tissue which will form roots (Duranz 1988). As previously mentioned, maturation is a complex process which incorporates numerous aspects of growth and development. Research has uncovered four main areas that may link maturation state and rooting; stem anatomy, rooting co-factor levels, endogenous rooting inhibitor levels, and the presence of preformed root initials (Clark 1982). Structural differences have, in some species, been cited as a factor affecting rooting, although such evidence is varied. White and Lovell (1984b) concluded that in *Agathus australis,* cuttings from older material contained abundant resin canals, and shlerenchyma branch traces, and that these reduced the amount of parenchyma tissue to such a low level that

potential primordial sites were no longer present preventing root formation. In contrast Davies *et al.,* (1982) suggested that the anatomical dissimilarities between juvenile and mature stems of *Ficus pumila* did not account for the differences in adventitious root formation. Perivascular sclereids (macrosclereids) were thicker in mature stems, but primordia penetrated these with relative ease. With some species, for example *Hedera helix*, juvenile tissues appear to contain higher levels of rooting co-factors than adult tissues (Clark 1982). Inhibitors have also been sited as a factor, as Paton *et al.,* (1970) found a direct relationship between the presence of an inhibitor and decreased rooting in *Eucalyptus grandis.* Caution is necessary when comparisons are made between plant species as there are large differences, for example generally the length of the juvenile phase increases in the following order, annual herbaceous plants, perennial herbaceous plants, shrubs, and trees (Hackett 1987).

Despite much work, there are still no clear answers to basic questions such as where in the cell or tissue, and how does maturation occur? Many workers have discussed whether stability is determined at the level of the individual cell, the entire apical meristem, or is due to correlative effects involving the whole plant (Bonga 1982a; Hackett 1985; Greenwood 1987; Pierik 1990). These three possible explanations have been termed cellular, structural and correlative respectively (Hackett 1985). Earlier workers proposed that increasing structural complexity and size of growing trees resulted in maturation (Borchert 1976). However this has recently been questioned (Pierik 1990). The evidence for all three is equivocal, the argument for an important role for meristems is fairly strong, for example when adult meristems are isolated and/or used in grafting experiments they are not easily altered (Bonga 1982a; Greenwood 1984). Greenwood (1987) later suggested that evidence exists to support all three possibilities. Using *Hedera helix*, he mentioned the stable behaviour of even extremely small grafted mature scions, which

nevertheless will exhibit juvenile characteristics after serial grafting or tissue culture. The suggestion was that maturation could result from an increase in the proportion of mature cells in the apical meristem. Conversely the previously mentioned methods of rejuvenation could promote relatively more rapid division of vestigial juvenile cells, which gradually increase relative to the mature proportion. Thus simultaneously a cellular basis (where some cells are irreversibly mature) plus explants being able to respond to external stimuli due to the remaining juvenile cells dividing could exist. There is still much work to be done in order to obtain a clearer understanding of this highly complex phenomenon.

## 1.4 Traditional methods of propagation used with eucalypts.

(i) Air layering.

Air layering is often used as a method of propagation where the formation of roots from cuttings is slow (Hartmann and Kester 1990). As the layer is attached to the parent plant a supply of water and nutrients is maintained during root formation. In addition, since eucalypts have an internal phloem, the shoot can be supplied with metabolites through this vascular tissue (Wilson and Bachelard 1975). There are several reports of young trees being successfully layered, but many mature trees are extremely difficult (Hartney 1980). Air layering is a labour intensive process and eucalypts are very slow to root when layered (Hartney 1980). Aerial roots have been recorded as naturally occurring from the stems of adult *E. camaldulensis, E. deglupta, E. robusta,* and several species of redgums (Hartney 1980; Cresswell *et al.,* 1982).

(ii) Grafting.

As grafting is very labour intensive and therefore an expensive operation, its application to forestry is limited to high value trees such as those used for seed orchards and ornamental horticulture (Hartney 1980). Grafting

has been used with eucalypts to preserve flower buds on the scions so that experimentally-controlled cross-pollinations can be done to establish seed orchards (Cresswell *et al.,* 1982). Graft incompatibility has been sited by many as a major problem with eucalypts (Hartney 1980; Mc Comb and Bennett 1986; Cresswell *et al.,* 1982). Examples of graft incompatibility include that in seed orchard trees of *E. grandis* in Australia (Burgess 1974) and in South Africa (van Wyk and Hodgson 1972; Hodgson 1977). In grafts of *E. deglupta* there were no incompatibility symptoms until shortly before the death of the tree which occurred several years later (Davidson 1977).

(iii) Stem cuttings.

The advantages of stem cuttings are that a large number of cuttings can be obtained from a single tree, the problem of graft incompatibility is avoided and it is less time consuming than grafting or layering and therefore cheaper. Cuttings taken from young eucalypt seedlings readily form roots (Cresswell *et al.,* 1982) and epicormic shoots have also been a good source of cuttings that form roots. Franclet (1956) found that with *E. camaldulensis* and *E. transcontinetalis,* cuttings rooted if taken from young seedlings (3 months to 1 year old) or epicormic shoots (four to five meters high and less than two years old) from 30 year old trees. However, cuttings from regular shoots of four to five year old trees could not be rooted. Further, cuttings from most mature eucalypts will not root (Paton *et al.,* 1970; Hartney 1980). However, the state of juvenility can be maintained by hedging, which has been utilised with *E. grandis* and various hybrids in Tunisia, the Congo and Brazil (Mc Comb and Bennett 1982). Shoots which sprout from the stump when a tree is felled can also have juvenile characteristics, including the ability to form roots (Mc Comb and Bennett 1986). To develop the desired clonal lines, trees with the required superior growth are felled and selection is made of those individuals that coppice well; rooting percentages of 80% and above have been reported for *E. grandis* (Campinhos and Ikemori 1977; Chaperon and Quillet 1977).

## 1.5 *In vitro* propagation of Eucalypts.

Cloning via organ culture has been successfully employed to obtain propagules from mature trees of some species for example, *Eucalyptus grandis* (Hartney 1980), however, most mature hardwoods and conifers remain difficult to micropropagate. Two of the greatest problems in the micropropagation of eucalypts are to obtain sterile material from field grown trees and the rooting of shoots from mature trees (De Fossard *et al.,* 1978 and Cresswell *et al.,* 1982). Another problem encountered with eucalypts is the formation of brown exudate resulting in the darkening of explants, callus and medium (Creswell and Nitsch 1975; Goncalves *et al.* 1979; Sita 1985). Propagation from nodal explants has taken one of two paths, one is direct induction of roots and shoots on an initial nodal explant (De Fossard 1974; De Fossard *et al.* 1977) and the other approach is to produce multiple shoots in aseptic cultures and then induce these shoots to form roots (De Fossard *et al.* 1977; Burger 1987).

### 1.5.1 Shoot cultures

Shoot multiplication has been achieved with many different *Eucalyptus* species of varying age, and a wide range of basal media (Table 3.1) have been used to induce this (Mc Comb *et al.* 1986). The multiplication rates are variable and are affected by factors such as the species, whether or not the shoots are juvenile or mature and the individual genotype (Mc Comb and Bennett 1986). Mostly this is carried out on a semi-solid medium (Sita and Rani 1985), but some use has been made of liquid media to stimulate shoot proliferation (Gupta *et al.* 1981; Rao and Venkatesara 1985; Gupta and Mascarenhas 1987).

### 1.5.2 Root induction

Induction of roots in mature *E. globulus* explants is difficult to achieve. Rooting media which have been used are similar to those used for

Table 3.1 Shoot multiplication media for *Eucalyptus* species.

| Species | Basal medium organic additives | Cytokinin (μM) | Auxin (μM) | Reference |
|---|---|---|---|---|
| *E. citriodora* | Murashige and Skoog (MS) | BAP 2.2-8.8 | NAA ? | Lakshmi (1979) |
| *E. citriodora*<br>*E. globulus*<br>*E. viminalis* | MS with Ca-Pantothenate (0.2 μM), Biotin (0.5 μM) | BAP 1.33 and K 0.93 or<br>BAP 2.2 and K 0.93 or<br>BAP 4.4 and K 0.93 | | Gupta *et al* (1981), Mascarenhas *et al.* (1982) |
| 12 species | 1/2 MS minerals (no vitamins) | BAP 1.0 | NAA 1.0 | Hartney (1982) |
| *E. diversicolor*<br>*E. marginata* | MS (no casein hydrolysate or glycine) | BAP 2.5 | NAA 1.25 | Modified from Bennett and Mc Comb (1982) |
| *E. nova-anglica*<br>*E. viminalis* | Modified Gresshoff and Doy | BAP 0.46 | IBA 0.05 | Mehra-Palta (1982) |

Table 3.1 continued.

| Species | Basal medium | Cytokinin (μM) | Auxin | References |
|---|---|---|---|---|
| | Organic additives | | (μM) | |
| *E. dalrympleana* | MS minerals (1/3 Ca) + high | BAP 0.5 | NAA 0.05 | Cresswell *et al.* (1982) |
| *E. delegatensis* | growth factors (see De | | | |
| *E. gunnii* | Fossard 1978) | | | |
| *E. pauciflora* | | | | |
| *E. ficifolia* | | | | |
| juvenile | modified De Fossard medium | BAP 2.0 | IBA 5.0 | De Fossard (1981) |
| mature | modified De Fossard medium | BAP 0.2 | IBA 5.0 | |
| E. | MS+0.1mg/l Ca panthothenate | BAP 0.5 mg/l | ---------- | Gupta and Mascarenhas (1987) |
| camaldulensis | + 0.1 mg/l Biotin | Kinetin 0.2 mg/l | | |

Abbreviations not used elsewhere : Ca = calcium; K= kinetin.

other genera, i.e. lowered mineral and higher auxin levels (Mc Comb and Wroth 1986; Sita and Rani 1985). Rooting is often achieved on media without vitamins (De Fossard *et al.,* 1978, De Fossard, 1981; Mc Comb and Bennett 1982; Hartney 1982), which is in contrast to shoot culture media which almost always contain vitamins and other organic addenda. The carry over effect of the shoot multiplication medium on subsequent rooting ability is often overlooked (Mc Comb *et al.,* 1986). Culture media sequences devised by Cresswell *et al.* (1982) and Depommier (1981) take this into account by including activated charcoal as well as gibberellic acid in the shoot elongation medium used before the root induction medium. Generally, cultures are placed in the dark, and then transferred to conditions similar to that used for shoot cultures.

Juvenile eucalyptus shoots usually root well (Franclet and Boulay 1982; Mc Comb *et al.* 1986). However there are exceptions, for example *Eucalyptus globulus* has given less than 30% rooting (Hartney 1982). Sankara and Venkateswara (1985) obtained 60% rooting with *in vitro* juvenile *Eucalyptus grandis* shoots, whereas only 35% of adult derived ones rooted. Various manipulations have been carried out to increase rooting frequency, for example several successive subcultures (Gupta *et al.* 1981), and successive grafting (Siniscaleo and Pavolettoni 1988) proved very effective. Examples of other strategies to improve rooting frequency include the use of coppice shoots (Mc Comb and Bennett 1986; De Fossard *et al.* 1977), spraying cytokinins on trunks to induce bud break (Mazelewski and Hackett 1979), and the use of juvenile parts on mature trees, for example lignotubers (Mazelewski and Hackett 1979). With difficult-to-root species such as *Eucalyptus globulus,* reports of rooting of mature explants frequently do not give all the essential details. The number of genotypes tested is important as the rooting treatment might be related to an easy-to-root genotype rather than a better rooting protocol for a wider range of genotypes in a population. Sankara and Venkateswara (1985) working with *Eucalyptus grandis* stated that the explants

Venkateswara (1985) working with *Eucalyptus grandis* stated that the explants were "mature", without further details of explant history or genotype. Gupta and Mascarenhas (1987) omitted reporting the number of genotypes used, although they did state the explant origin and that the trees were mature "elite" fast-growing. Two reports of micropropagation of mature *E. globulus* had rooting percentages of 40-60% (Mascarenhas *et al.* 1982; Gupta and Mascarenhas (1987), the number of genotypes used was not presented in either of the two reports, which makes interpretation of the data rather difficult. From the literature it seems that with mature *E. globulus* only a limited number of genotypes can be vegetatively propagated.

## 1.6 Auxin and Adventitious Root Formation.

Monceau (1758) suggested that the formation of adventitious roots on stems was due to the downward movement of sap, and Sachs (1880, 1882) proposed the existence of an active substance which formed in the leaves and buds and accumulated in the base of stem cuttings. Van der Lek (1925), working with willow, poplar and grape demonstrated that active buds produced a substance(s) which promoted rooting, since rooting was inhibited after an incision was made through the vascular system between the bud and the site of root initiation. Went (1929) noted that the leaves and buds on stem cuttings of *Acalypha wilkesiana* Muell. Arg. also promoted rooting and assumed that they were the source of the hypothetical 'root-forming phytohormone' (rhizocaline). To make his studies of rhizocaline quantitative Went, (1934) established a standard bioassay procedure using etiolated pea hypocotyls. Went proposed the 'rhizocaline unit', each of which would produce one root above the control value when tested under the conditions specified. Thimann and Went (1934) discovered that 'heteroauxin', isolated from urine (Kogl *et al.*, 1934) stimulated adventitious rooting in the pea test developed for rhizocaline.

Following the chemical identity of IAA, its ability to promote adventitious root formation was soon established (Cooper 1935, Thimann and Koepfli 1935). Later work, in which the response of cuttings to applied auxins was variable, led to the proposal that other substances were required for root formation (Cooper 1938, Went 1938 Thimann and Delisle, 1939). Compiling a large list of the rooting responses of a wide range of species to applied auxins, Audus (1959) stressed the positive response of the majority of species, but suggested that auxin was one of the determinants whose effectiveness may be affected by endogenous factors such as nutrients and other plant growth substances.

Auxin applications have proved very effective in promoting ARF and it is for this reason that it has received considerable attention amongst plant physiologists working in propagation research. Auxin is generally accepted to play a central role in ARF in the so-called easy-to-root cuttings. Hartmann and Kester (1990) describe two patterns of ARF in cuttings for both herbaceous and woody species; direct and indirect root formation. In addition in certain species pre-formed root primordia exist, for example in *Salix fragilis* (Lovell and White, 1986).

1.6.1 Preformed root primordia.

Root initials develop in intact stems of *S. fragilis* at node 4, which is the transition region separating the zones of rapid growth and maturation (Haissig 1970). They are located in the leaf gaps where ray cells develop large nuclei, prominent nucleoli and dense cytoplasms. Cell number increases due to cell divisions occurring in all planes but with little enlargement of the daughter cells (Haissig 1970). Following severance, further cell divisions give rise to a structure of similar appearance to a young root primordium which is about to push out into the cortex (Carlson 1938). The structure can remain dormant, at a primordium initial stage, over winter or even for a number of years, in the absence of severance.

1.6.2 Direct and indirect root formation.

Hartmann and Kester (1990) describe two patterns of ARF in cuttings for both herbaceous and woody cuttings. The direct pattern of root formation involves direct formation of root primordia from cells associated with or in close proximity to the vascular system. The indirect pattern of root formation involves an interim period of undifferentiated cell division. These cell divisions are usually initiated in parenchyma or epidermal cells. Certain cells within these subsequent cell divisions eventually organise to initiate an adventitious root primordium. (Hartmann and Kester 1990). Herbaceous species and easy-to-root woody species generally root through the direct pattern of ARF (Hartmann and Kester 1990). With many difficult-to-root species, root formation can occur via both the direct and indirect pattern (Davies *et al.*, 1982; Gronroos and Arnold 1987; Geneve 1991). *Hedera helix* has relatively stable ontogenetic phases, in the juvenile phase the cuttings are easy-to-root, and in the mature phase cuttings are difficult-to-root (Hackett *et al.*, 1988). It has also been shown in *H. helix* that the easy-to-root juvenile phase cuttings root mainly via the direct pattern of ARF, whilst difficult-to-root mature phase cuttings proceed via the indirect pattern of ARF (Girouard 1967a,b; Geneve *et al.*, 1988).

Most workers agree that ARF consists of a number of distinct stages, and much work has been carried out to define these distinct stages in ARF (Davies *et al.*, 1982; Lovell and White, 1986 and Hartmann and Kester 1990), which essentially include:

(1) Dedifferentiation and the formation of a new meristematic locus .

(2) Early cell divisions producing a cluster of cells which are radially symmetrical.

(3) Later cell divisions to form an organised determined root meristem.

(4) Root formation by extension growth of cells produced by the meristem.

(Doré, 1965 and Girouard, 1967a)

Work has shown that the different stages of ARF have different auxin requirements (Jarvis & Shaheed 1986; Gaspar and Coumans 1987 and Blakesley *et al.*, 1991a). Evidence can be drawn from both studies on applied auxin and endogenous levels.

## 1.7 The role of applied auxin in ARF.

IAA (Figure 1.1) is synthesized in shoot apices and lateral buds (Sembdner *et al.*, 1980; Nordström and Eliasson, 1991). Auxin movement is preferentially basipetal (Morris *et al.*, 1969; Jacobs, 1979). Suttle (1991) working with etiolated *Helianthus* hypocotyls found that there was a decline in basipetal IAA movement with increasing age, the correlation between increasing plant age and decline in rooting ability is well documented (Davies *et al.*, 1982; Greenwood 1987). Decapitation, in sunflower hypocotyls, (Liu and Reid 1992) or the use of anti-auxins, in avocado microcuttings, (Garcia-Gomez *et al.*, 1994) leads to a reduction in root primordia number.

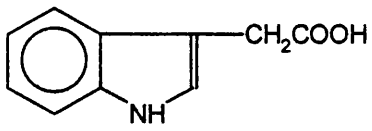
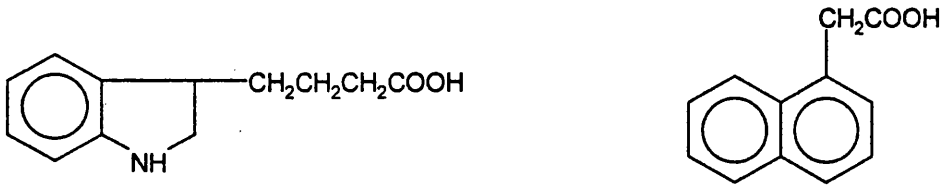



Figure 1.1 Structure of IAA.

The most successful method for induction of ARF in woody species has been applications of synthetic auxins, indolebutyric acid (IBA) and 1-napthaleneacetic acid (NAA) to *in vivo* stem cuttings, and shoots cultured *in vitro* (Jarvis, 1986; Alvarez *et al.*,1989a; Garcia-Gomez *et al.*, 1994). It has been suggested that the increased efficiency of the synthetic auxins IBA and NAA over IAA for root initiation may be related to differences in the rates at which these auxins are metabolised in plant tissues (Jarvis 1986). However,

Wiesman *et al.*, (1988) found that the rate of metabolism of IBA resembled that of IAA. The type of auxin has been shown to play an important role, for example in the morphology of the root system which subsequently emerges (Gorst *et al.* 1983). Using *in vitro Eucalyptus ficifolia*, Gorst *et al.*, (1983) reported that all explants on a medium containing IBA produced a root system consisting of many short roots, with extensive lateral development. IAA application also resulted in a rather "stunted" root system, but lateral development was minimal.




IBA NAA

Figure 1.2 Structure of IBA and NAA.

James and Thurbon (1981 a & b) showed that in *Malus pumila* root stocks which were genetically related, but differed in their rooting ability, the difficult-to-root rootstock (M9) had a higher auxin requirement than the easy-to-root rootstock (M26). The differential response led workers to suggest that differences in the rooting ability between M9 and M26 resulted from differences in endogenous auxin levels (Le 1985). The role of applied auxin also differs at different stages of ARF (Erikson and Mohamed 1974; Mohammed and Eriksen 1974; Kantharay *et al.* 1979; Liu and Reed 1992). These workers showed that it is the early stages of cell division and organisation of the primordium which require high auxin levels and that the later stages do not have a high auxin requirement. Nahlawi and Howard (1973) found that plum rootstock cuttings rooted better with a single application of IBA than with repeated applications. Indeed, Thimann (1936) showed that IAA

can be inhibitory to root development in all but the lowest concentrations. In a more detailed study, Smith and Thorpe (1975a) working with ARF in *Pinus radiata* hypocotyls found that IBA was necessary over the 4 day period prior to the formation of a meristematic locus and in the next two days during which the first cell divisions occurred, but not for the subsequent stages of root formation. The period where a positive response in ARF to auxin occurs has been termed the auxin 'sensitive' phase (James 1983). Although the length of the auxin sensitive phase is, to a certain extent dependent on the type and concentration of the auxin and plant species this evidence suggests an early role for endogenous auxin in the initiation of root primordia (Blakesley *et al.*, 1991a).

## 1.8 The role of endogenous IAA in ARF.

Much of the early work on the role of endogenous IAA levels utilised bioassays for the quantification of IAA, but in recent years a number of workers have attempted to correlate endogenous IAA levels with ARF in cuttings using reliable physico-chemical techniques (Alvarez *et al.* 1982a; Noiton *et al.* 1992a). Quantification of endogenous auxin levels has been carried out with respect to the rooting of different species (Bose *et al.* 1973), cultivars of the same species (Kracke *et al.* 1981; Alvarez *et al.* 1989b), seasonal variations (Vieitez and Pena 1968; Blakesley *et al.* 1991a), and age differences (Hengst 1959). Largely utilising bioassays, the early work on endogenous auxin levels and adventitious rooting often considered the auxin status of the cutting at the time of excision, but such systems produced contradictory results. In *Dahlia* (Biran and Halvey 1973), *Rhododendron* (Wu and Barnes 1981), and *Chrysanthemum* (Stolz 1968) the capacity of cuttings to form adventitious roots was not correlated with endogenous levels of IAA in the mother plants, i.e. the difference between easy-to-root and difficult-to-root

cultivars could not be explained by endogenous IAA level. In contrast with *Hibiscus* (Bose *et al.* 1973) and *Vitis* (Bartolini *et al.* 1986; Kracke *et al.* 1981), there was a positive correlation between the ease of rooting and endogenous auxin levels. Kracke *et al.* (1981) using two cultivars of grape rootstock did not, however, report the bioassay used for IAA estimation.

The more closely related the tissue being used, the less chance there is of genetic causes for differing auxin measurements. Hengst (1959), showed that the variation in rooting in *Streptocarpus* leaves was closely related to variation in auxin content of the whole plant. Recently, Bouza *et al.* (1994), using a combined HPLC-ELISA technique reported that *Peony* explants differed in their rooting capacity depending on the origin and subculture duration and that there was a positive correlation between rooting capacity and endogenous auxin level. Alvarez *et al.* (1989a) reported a positive correlation between auxin levels in the rooting ability of M9 and M26 apple rootstock. In the bases of the easy-to-root (M26) shoots there was significantly more free IAA than in similar difficult-to-root (M9) shoots, whereas in apical sections of both lines free IAA levels were comparable. In addition to higher free IAA levels being associated with higher rooting ability, a greater proportion of IAA was present as a conjugate in the difficult-to-root shoots. However, this is in contrast to Welander and Snygg (1987) working with apple rootstocks M26 and A2, and with Noiton *et al.* (1992b) working with 'Johnothan' apple in which different rooting ability was generated by the number of subcultures *in vitro*.

Several workers have investigated seasonal rooting patterns and associated auxin levels and found a positive correlation between the two. Blakesley *et al.*, (1991b) used *Cotinus coggygria* which rooted well in the spring, and very poorly in the summer and autumn. The levels of IAA in the rooting zone (stem base) of cuttings taken in the spring was significantly higher than in the summer. Like Alvarez *et al.*, (1989a) they also found a higher ratio

of conjugated IAA to total IAA associated with the poorer rooters. IAA has been associated with the onset of cambial activity (Little and Wareing 1981; Sundberg *et al.* 1991) and with increased growth rate (Bandurski *et al.*, 1977), so it might be expected that in the summer cuttings the concentration of free IAA would be less than in the spring.

### 1.8.1 Endogenous IAA levels during the rooting process.

The timing of events is critical as one needs to be confident about which stages in ARF are actually being monitored. Nordström and Eliasson (1991) working with pea cuttings, monitored IAA and indole-3-acetylaspartic acid during ARF. Identification utilised GC-MS and quantification, an HPLC-spectrofluorophotometric detection system. They concluded that root initiation may occur without increased IAA levels in the rooting zone. For the timing of the events of ARF, they referred to Bollmark *et al.*, (1988) who reported that the first mitoses take place 1-2 days after cutting excision. The organisation and growth of root primordia begin during the next few days and the first visible roots appear 5 days after cutting excision. Their first harvest occurred 1 day after cutting excision. Hausmann (1993), using the same HPLC detection system as Nordström and Eliasson (1991), worked with *in vitro*-raised poplar shoots and concluded that a decrease in IAA in the shoots preceded rooting. This was considered as corresponding to the initiative stage of rooting. Preceding this was a peak in IAA activity and it was concluded that this might initiate the inductive stage of ARF. Rooting was uniform on the rooting medium and 100% of the shoots had rooted by the sixteenth day; however no histological work was reported. Recently, Garcia-Gomez *et al.*, (1994) working with avocado microcuttings found endogenous IAA levels in the base (first cm) and apical part (rest of the cutting) remained constant when the cuttings were rooted in the absence of exogenous auxin. After 3 days on the

rooting medium some nuclei appeared densely stained, coinciding with the first harvest for analysis of endogenous IAA.

Several independent workers have reported that after a transient rise in free IAA associated with the induction period of ARF, a decline in auxin levels coincided with early cytological events (Blakesley, 1984; Moncousin *et al.*, 1988; Gaspar *et al.*, 1990). In addition there is evidence of a positive correlation between the site of ARF and localisation of IAA levels. Blakesley (1984) working with *Phaseolus aureus* hypocotyl cuttings found that the transient rise in IAA level which occurred prior to early visible events, only took place in the basal section of the hypocotyls where the roots form. Moncousin *et al.*, (1989) working with an *in vitro* grapevine and using ELISA also detected an early transient IAA peak in the basal region of the cuttings, which was not found in the apical part of the shoot. That endogenous IAA may be of importance as a promoter of ARF only after the end of the induction phase has been suggested (Norcini *et al.* 1985). Using ELISA techniques an increase in IAA was detected in whole terminal cuttings of chrysanthemum (Weigel *et al.* 1984); here IAA was recorded as increasing until the first adventitious roots penetrated the epidermis. Label *et al.*, (1989) working with *in vitro* rooting of *Prunus avium* explants, and Maldiney *et al.*, (1986) working with hypocotyl cuttings of Craigella and Craigella lateral suppressor tomatoes, both using ELISA found similar results suggesting that there was a concomitant rise in IAA and the primary event of root initiation.

In cuttings of easy-to-root cultivars of *Hibiscus* and *Bougainvillea*, endogenous IAA levels were reported to decline during root formation (Bose *et al.*, 1973). Working with *Sequoiadendron giganteum* cuttings Berthon *et al.*, (1989) also found root initiation occurred with a reduction in IAA levels.

From the large amount of work which has been carried out on IAA analysis during ARF there still seems to be some debate. To avoid confusion it is important to have a clear understanding of the sequence of histological

events and to insure that IAA analysis is timed suitably so that the possibility of missing phases does not occur. For reliable results in IAA analysis, unequivocal physico-chemical techniques, (Sandberg *et al.,* 1987a), should be employed.

## 1.9 The role of IBA.

As previously mentioned in the work from auxin applications, IBA has been a very successful treatment for the promotion of ARF. The role of IBA still has several unanswered questions. Following uptake, IBA can be conjugated with amino acids (Wiesman *et al.* 1989; Nordström *et al.* 1991). Comparable to IAA conjugates, the IBA conjugates form a potential source of free IAA and are not physiologically active themselves (Cohen and Bandurski 1982; Wiesman *et al.* 1989). IBA has also been reported to be converted into IAA (Epstein and Lavee 1984; Alvarez *et al.* 1989a; Van der Krieken *et al.*, 1992a, b 1993). The metabolism of IBA, unlike that of IAA, has not received much attention. Some workers have suggested that a possible mode of action of IBA is via conversion into IAA (Epstein and Lavee 1984; Van der Krieken *et al.* 1993), this is contrast to IBA exerting a direct effect (Nordström *et al.* 1991).

## 1.10 Competence and determination.

The terms competence and determination have both been adapted from animal to plant studies. Competence in plants is generally defined as a state of reactivity of cells to respond to specific stimuli (Meins, 1986). Here competence for root formation will be defined as the ability of cells within a tissue to respond to specific root-inducing stimuli by the formation of roots.

Once exposed to an inducer the competent cells may become determined for root formation. Determination can be defined as the commitment of cells to a specific developmental fate (Meins and Binns 1979). Therefore, once a cell(s) has received a signal for root formation, commitment to root formation will remain even after removal of the root-inducing stimuli. The state of determination is usually deduced by experimental manipulation of cells, tissues or organs (Meins and Binns 1979). Frequently the manipulation used for studies of determination for rhizonogenesis has involved transfer of tissue explants from media containing a root-inducing factor(s), the so-called root-inducing medium (RIM), to a medium without the factor(s), (basal medium), and counting the number of roots after a certain period of time. Frequently the root-inducing factor is an auxin, sometimes combined with a relatively low level of cytokinin. An auxin requirement for competence and determination of tissue explants to form roots has been suggested by Bonnett and Torrey, (1965) and Lyndon (1990), with *Convolvulus*. With the apparent ease of manipulating organogenesis in tissue culture conditions (Skoog and Miller, 1957), surprisingly few studies have been reported on the competence and determination of tissues for root formation (Mc Daniel, 1984).

Mohnen (1994) highlighted several systems for studying competence and determination for ARF, and discussed work on apple shoot cultures (James and Thurbon, 1979; Sriskandarajah *et al.*, 1982; James, 1983), *Hedera helix* leaf cuttings and debladed petioles (Geneve *et al.*, 1988; Geneve *et al.*, 1991), callus (Walker *et al.*, 1979), leaf explants (Christianson and Warnick, 1985; Warnick 1992) and the use of thin cell-layer explants (Mohnen 1994). As mentioned earlier, an advantage of the *H. helix* system is that of relatively stable ontogenetic phases, where the juvenile phase is easy-to-root and the mature phase is difficult-to-root (Hackett *et al.*, 1988). Geneve *et al.*, (1991) used sterilised debladed petioles from juvenile- and mature-phase *H. helix*, incubated on a medium containing 100 μmol α-naphthalenacetic acid (plus

1mM of the polyamine biosynthesis inhibitor, difluoromethylarginine). This work demonstrated that culture of juvenile petioles on NAA-containing medium for as little as one day permitted some root formation, whilst longer exposures up to eighteen days yielded larger numbers of roots. Therefore, as little as one day was sufficient to allow juvenile petioles to become determined for root formation. Since root meristems were first formed after approximately 12 days of culture, determination for root formation preceded meristem formation. Root meristems in juvenile petioles originated in cortical parenchyma cells adjacent to vascular bundles. In contrast, the few roots which formed in mature petioles developed from callus that developed from cortical cell division. Figure 1.3 shows the proposed scheme for adventitious root primordium formation through the direct or indirect pattern of organ formation (from Geneve 1991). The data supported the use of the terms "competent root-forming cells" and "induced competent root-forming cells" to describe the target cells for the initial events of root formation for the direct and indirect patterns of rooting respectively. Determination of explants for root formation is a lengthy process, usually requiring days rather than hours. This is sufficient time to allow cell division to occur, but there is still uncertainty whether there is a correlation between cell division and determination (Mohnen, 1994). In certain cases tissues taken directly from the plant may not be competent to respond to root-inducing factors, and attention may need to be paid to the initial attainment of competence in order to maximise rooting potential (Christianson and Warnick, 1985).

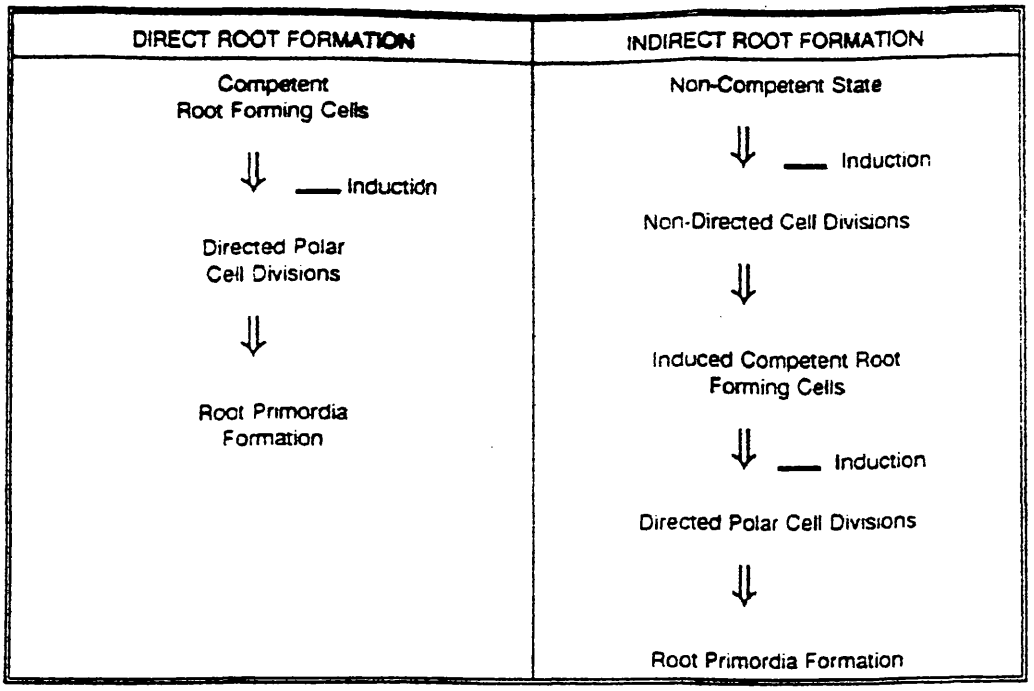



Figure 1.3 Proposed scheme for adventitious root primordium formation in *Hedera helix* through the direct or indirect pattern of organ formation (From Geneve, 1991).

Experimental approaches that can effect the competence for ARF include the growth of shoots in darkness (etiolation) and the exclusion of light from tissue that was initially light-grown (blanching) (Harrison-Murray, 1982). A general or localised exclusion of light from shoot tissue during the early stages of growth from apical or axillary buds can influence the competence for adventitious root formation in cuttings taken from the treated shoots (Gardner, 1936; Herman and Hess, 1963; Harrison-Murray, 1982; Maynard and Bassuk, 1988). Howard (1980, 1981, 1984, 1985), using apple rootstock M.9., has studied the effects of dark treatments on ARF. These studies indicated that there was, on average a seven-fold increase in the rooting percentage for auxin

treated cuttings that were etiolated initially, but acclimated in light for two weeks (78%), as compared to auxin treated, light-grown control cuttings (11%) (Harrison-Murray, 1982). Although blanching light-grown shoots was significantly less effective than etiolation in changing or maintaining competence (Howard 1981), the initial growth of shoots under heavy shading has been shown to be as effective as complete darkness in influencing the rooting potential of M.9. cuttings (Howard, 1984, 1985). The experiments by Howard with apple rootstock M.9. together with work on avocado (Frolich, 1961), indicate that the effect of etiolation is not ephemeral, but can persist for many months subsequent to the exposure of light. The persistent effect of etiolation with subsequent exposure to light on the rooting of M.9. hardwood cuttings nine months later (Harrison-Murray, 1982), suggests there is a fundamental change that is not related to the level of endogenous auxin, or other transient metabolic effects.

## 1.11 Sensitivity and evidence from transgenic plants.

Trewavas (1981) brought to the fore the theory that in many cases sensitivity (responsiveness) to plant growth substances is more important than absolute concentrations of plant growth substances. At the time more concern was placed on establishing the importance of sensitivity than in discussing its measurement or theoretical basis. The discussion by Trewavas (1981) stimulated a greater awareness of sensitivity which was reflected in several publications that followed (Cleland 1983; Salisbury and Ross 1985; Moore 1989; Guern 1987; Davies 1987). In a later paper Trewavas (1991) discussed the actual measurement and theoretical basis of sensitivity. His criteria for an unambiguous measurement of growth substance sensitivity are:

i To manipulate growth substance concentrations at the endogenous level in order to avoid artefact.

ii Constraint on experimental manipulation with the preferable use of whole plants.

iii Methods which assess the plant growth substance contribution to control when a number of other factors also contribute.

The conditions are not easy to meet, but would allow the measurement of sensitivity at the plant growth substance level which Trewavas (1991) defined as control strength. Control strength requires knowledge of the fractional change in response (R) and the fractional change in endogenous growth substance concentration (C). The value of the control strength is obtained by dividing the change in R by the change in C. There are very few plant growth substance studies which permit control strength to be estimated. Two sets of data which lend themselves to possible estimates of control strength were published by Ingram *et al.* (1986) and Lenton and Hedden (1987). In both cases, the gibberellin control strength for leaf growth and tall pea stem growth can be estimated to be no greater than 0.05 (0.95 of the control is elsewhere). One explanation of this is that plants growing near their maximal rates will have their control of growth shared amongst many different molecules (Trewavas 1991). Under different circumstances such as shade conditions these control strengths could easily change (Trewavas 1986).

Hairy root disease and crown gall disease are incited by *Agrobacterium rhizogenes* and *A. tumefaciens* respectively. Virulent *A. rhizogenes* and *A. tumefaciens* bacteria harbour a Ri (root inducing) plasmid and a Ti (tumour inducing) plasmid respectively. These plasmids are of interest as they contain genes involved in the biosynthesis of plant growth substances. In addition transfer of certain regions of the Ri plasmid has been reported to confer increased sensitivity to auxin in plant tissue (Maurel *et al.*, 1991; Shen *et al.*, 1988 ). An abundant proliferation of roots occurs at the site of inoculation when the Ri T-DNA is expressed. Ri plasmids are classified according to the type of opine which is synthesised in transformed tissue. Following

incorporation of Ri plasmid T-DNA into the plant genome three opines have been identified; mannopine, agropine and cucumopine. Agropine type Ri plasmids differ in that they have two T-DNA regions, $T_L$ and $T_R$. Strains of *A. rhizogenes* which synthesise the other two types of opine have just one T-DNA region which shares considerable homology with the $T_L$ region of the agropine strains. Hairy root disease is also characteristic of plant tissue transformed by the mannopine-type *A. rhizogenes* which does not contain the $T_R$ region. The $T_L$ region of the Ri plasmid stimulates root formation independently of the transfer and expression of the $T_R$ T-DNA genes. The *A. rhizogenes* T-DNA agropine-type Ri plasmid pRiA4 was characterised by White *et al.*, (1985). Four genetic loci were identified (*rol*A, *rol*B, *rol*C and *rol*D) which were identified according to the tumour morphology observed when insertions were made into each genetic locus. *Rol* gene loci have since been studied by many research groups (Shen *et al.*, 1988, Estruch *et al.*, 1991, Maurel *et al.*, 1991, Schmulling *et al.*, 1993).

Enhanced auxin levels may be important for auxin sensitivity through the *rolB* gene coding an enzyme (β-glucosidase) capable of hydrolysing indole-β-glucosides (Estruch *et al.*, 1991). However the substrate tested for the *rolB* gene enzyme by Estruch *et al.*, (1991) was not an auxin, and if the enzyme is able to hydrolyse auxin conjugates *in vivo* it could be useful for the manipulation of endogenous auxin (Blakesley, 1994). β-glucosidase was not able to hydrolyse IAA-glucose conjugates (Spena *et al.*, 1993). Specific expression of the *rol*-B gene increased the free IAA content in transgenic anthers (Spena *et al.*, 1992), although expression of the gene appears not to influence the overall rate of IAA biosynthesis (Nilsson *et al.*, 1993). Using *Lotus corniculatus* root tips Shen *et al.*,(1988) looked at the physiological properties of protoplasts from transformed (*A. rhizogenes*) and untransformed material. Hairy root transformed cells were $10^2$ to $10^3$ times more sensitive to the effects of auxin when compared to the untransformed cells. Increased

sensitivity being an early cellular event, possibly involving the reception or transduction of the hormone signal was suggested by the authors. Barbier-Brygoo *et al.*, (1990) found that each of the single genes *rolA, rolB* and *rolC* was able to confer increased sensitivity to auxin in transformed protoplasts, *rol*B being the most effective (Maurel *et al.*, 1991). The apical region of 7 week old transformed and *rol-A* tobacco plants contained about half the endogenous IAA concentration present in the apical region of the control plants (Prinsen *et al.*, 1994).The detailed kinetic hormonal analyses (Prinsen *et al.*, 1994) emphasised the relevance of varying hormone levels in the shoot apical region on the developmental pattern of both transgenic and normal plants. Maurel *et al.*, (1991) postulated that increased sensitivity to auxin could be a major determinant for root differentiation.

The potential value of studies on sensitivity and the use of transgenic plant tissue has been suggested in several papers (Blakesley and Chaldecott 1993; Hamill and Chandler 1994). The study of the structure of the genes involved in the biosynthesis and metabolism of plant growth regulators, and in apparent sensitivity to plant growth regulators, could provide information on the role of IAA in root initiation. Incorporation of auxin biosynthetic genes from *A. rhizogenes* or *A. tumefaciens* may also permit manipulation of endogenous IAA levels.

## 1.12 Plant hormone analysis.

The analysis of plant hormones involves the following basic processes; extraction, purification, qualitative and quantitative analysis. Analysis of plant hormones has greatly improved in recent years due to better instrumentation (Sandberg *et al.*, 1987a) and improved isotopically labelled compounds (Cohen *et al.*, 1986).

1.12.1 Extraction.

Addition of a suitable isotopically labelled internal standard to the sample, permits the loss of IAA during extraction and purification to be corrected for without knowing the recovery (Cohen *et al.*, 1986). However, in order to obtain an accurate IAA estimate, firstly the isotopically labelled compound should equilibrate with the endogenous IAA pool, otherwise an underestimate will occur. Secondly, IAA must not be formed during sample preparation, or an overestimate of IAA will occur. IAA formation during sample preparation has been documented, (Bandurski *et al.*, 1977; Ernstsen *et al.*, 1986; Sundberg 1990), but can be reduced by minimising sample handling, using the shortest extraction time for equilibrating the internal standard with endogenous IAA, and by adding an antioxidant (Nakajima and Yamazaki 1979; Lino *et al.*, 1980; Ernstsen *et al.* 1986).

## 1.13 Purification.

A wide range of methods are available for the purification of indoles in plant extracts. Purification often includes more than one of the following methods, solvent partitioning, polyvinylpyrrolidone (PVP), anion-exchange resins, Sep-Pak cartridges and HPLC. Generally the greatest reductions in dry weight are obtained when the individual purification procedures display distinctly different separatory mechanisms (Reeve and Crozier. 1978).

1.13.1 Solvent partitioning.

The initial purification step, especially with large amounts of material and/or with highly pigmented plant tissue, traditionally included solvent partitioning (Epstein and Cohen 1981; Cohen *et al.*, 1986; Sundberg *et al.*, 1991; Li *et al.*, 1992). Tissue is partitioned between an aqueous phase and an immiscible organic solvent. The distribution of IAA (and other ionisable

molecules) is influenced by its $pK_a$ and the pH of the aqueous phase, in an uncharged form it migrates into the organic phase (Yokota *et al.*, 1980). Numerous solvent partitioning schemes have been reported in the literature. Figure 1.4 (from Sandberg *et al.*, 1987a) shows a general partitioning scheme in which the extracts are firstly dissolved in 0.1 M phosphate buffer (pH 8) then partitioned against ethyl acetate. Frequently an antioxidant, such as butylated hydroxytoluene, is incorporated in the solvents used for extraction and partitioning, to help suppress the problem of break down of indole-3-pyruvic acid (IPyA) into IAA (Sandberg *et al.*, 1987a). There has been a general move away from the use of solvent partitioning procedures for IAA ( Sandberg *et al.*, 1984; Sundberg *et al.*, 1985; Nordström and Eliasson 1991) as there are more rapid and effective methods available (Sandberg *et al.*, 1987a), and the amount of plant tissue necessary for analysis has decreased (Epstein and Cohen 1981; Prinsen *et al.*, 1992).

Acidic indoles, such as IAA, remain in the aqueous phase when partitioned against ethyl acetate in pH 8 phosphate buffer (Figure 1.4). However, even when IAA is the only compound of interest, the pH8 partitioning step is frequently included as this removes significant amounts of impurities which would otherwise contaminate the acidic ethyl acetate fraction (Li *et al* 1992). When analysis is concerned with a specific acidic indole, its $K_d$ may be such that it can be extracted into diethyl ether rather than ethyl acetate. Diethyl ether is less polar than ethyl acetate and therefore will yield cleaner samples, examples where IAA has been partitioned into diethyl ether include the work of Knegt and Bruinsma (1973) and Cohen *et al.*, (1986).

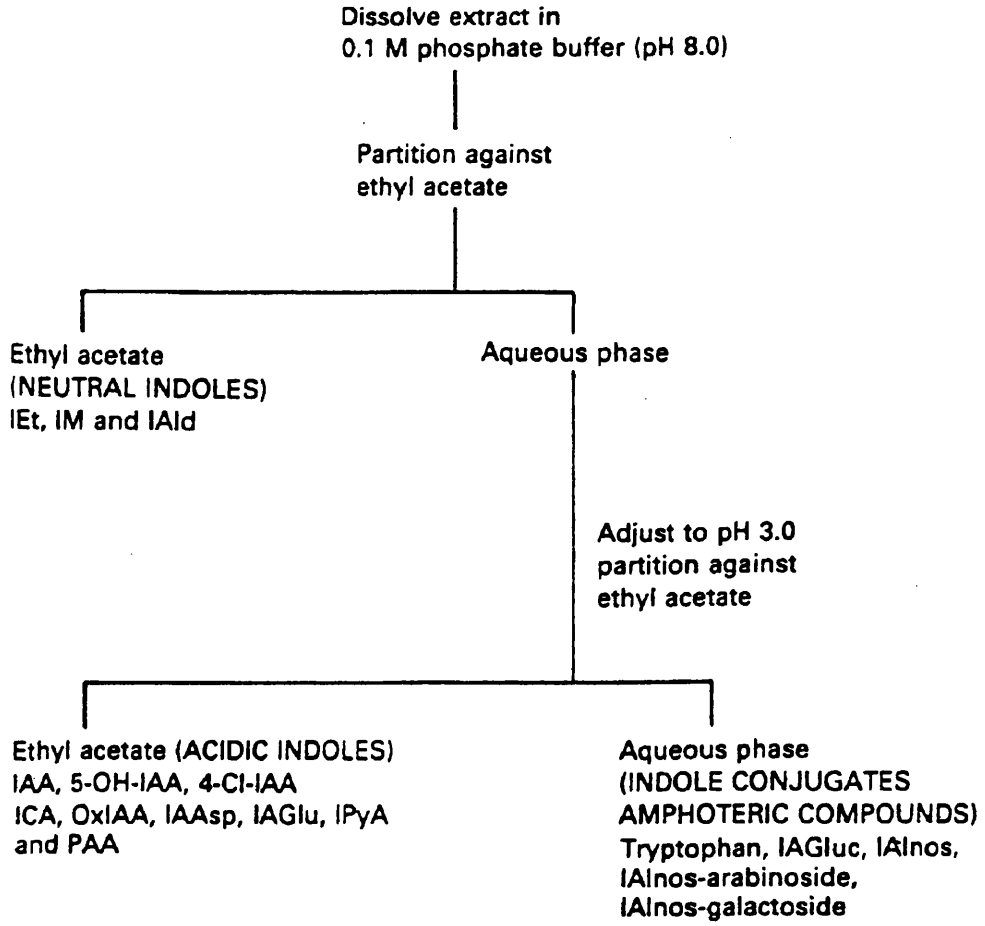



Figure 1.4 Partitioning procedures for the separation of indoles into neutral, acidic and conjugate fractions (Sandberg *et al.*, 1987a).

1.13.2 Polyvinylpyrrolidone (PVP).

PVP has been used extensively for the purification of plant hormones (Sandberg *et al.*, 1981, 1987a) because it is a simple and flexible technique, in that column sizes can be adjusted to accommodate a wide variety of samples and it yields a high recovery. A key element in the effectiveness of PVP in purification protocols is its property of retention of phenolic compounds by hydrogen bonding at low pH (Yokota *et al.*, 1980; Sandberg *et al.*, 1987a). The retention of auxins by PVP is a pH-dependent process. Indoles are highly retained when chromatographed in an acidic buffer (Yokota *et al.*, 1980; Sandberg *et al.*, 1987a). Purification of auxins in plant extracts using PVP

column chromatography has often been reported (Mc Dougall and Hillman 1978; Sandberg *et al.*, 1981; Blakesley *et al* 1991 b).

1.13.3 Ion-exchange chromatography.

Anion-exchange resins, for example Sephadex QAE-25, retain acidic compounds. This property can be utilised effectively in the purification of indolic compounds. When purifying plant samples it is important not to overload the exchange capacity of the column. Reservoirs containing the ion-exchange resin can be coupled to Sep-Pak cartridge systems.

1.13.4 Sep-Pak cartridge systems.

Recently several types of small prepacked cartridge systems containing various chromatographic materials have become available. The Sep-Pak system from Waters Associates was one of the first and can be obtained with either silica gel or a $C_{18}$-coated silica support. One use is as an off-line precolumn prior to HLPC (on-line HPLC precolumns often produce some degree of band spreading). A second use is as a combined purification/concentration step; in this mode the sample is applied to the cartridge in a weak solvent so that the compound of interest is adsorbed. Next the cartridge is washed to remove impurities before the solvent strength is increased eluting the compound of interest. The use of silica and $C_{18}$ cartridges can be combined in a number of ways as described by Sandberg *et al.*, (1987a).

1.13.5 High performance liquid chromatography

High performance liquid chromatography (HPLC) has been used by scientists from many fields. As reversed-phase HPLC has been used to obtain data in this thesis it is only this system which will be described in any detail. In this form the stationary phase is organic, usually a long alkyl chain and the mobile phase is primarily water but with an organic modifier. Ionic samples may be separated on this type of column by either of two techniques:

Ion suppression: if the sample is a weak acid or base, its ionisation can be suppressed by changing the pH of the mobile phase so that the solute becomes less polar.

An alternative is ion pairing, where the sample solute (a strong acid or base), can be paired with a counter ion added to the mobile phase, to form an effectively neutral ion pair.

Columns are eluted either isocratically or with a gradient of increasing amounts of organic solvent, for example methanol or acetonitrile, in water or an aqueous buffer. Commercial supports are produced with different types of silica gel, and variations exist in the procedures used in to bond the stationary phase, the degree of stationary phase loading and the effectiveness with which residual active sites are encapped. As a consequence, even different batches of the same material do not show identical retention characteristics. Exact conditions for a particular analysis have to be optimised 'on-site'.

Wurst *et al.*, (1980, 1984) and Jensen (1982) reported detailed studies of reversed-phase HPLC of indoles. The pH of the mobile phase had a marked effect on acids, for example IAA eluted much more rapidly at pH 7 than pH 3.5 (Jensen 1982). At pH 7 carboxyl groups are ionised, and in the reversed-phase mode charged molecules are distributed preferentially into the more polar aqueous mobile phase. In practice, acidic indoles are usually analysed in an acidic mobile phase in an undissociated form, (ion-suppression reversed-phase HPLC (Sandberg *et al.*, 1987a).

## 1.14 HPLC detection systems.

### 1.14.1 Absorbance detectors.

U.V. absorbance monitors operating at 280nm can be used to detect most indoles in HPLC eludates. The limit of detection using reverse phase analysis of IAA is in the low nano-gram range (Crozier *et al.*, 1980). Scanning

diode-array detectors are able to record whole spectra on HPLC peaks, and even sections of HPLC peaks, as they pass through a flow cell (Horgan 1987). Due to the large number of compounds that elicit a response at $A_{280}$, an absorbance monitor only offers a low degree of selectivity when analysing endogenous indoles (Sandberg *et al.*, 1987a).

1.14.2 Fluorescence detectors.

Since IAA is naturally fluorescent, with excitation and emission maxima at 280nm and 350nm, respectively, it can be selectively monitored with a spectrophotofluorimeter (Burnett and Audus 1964; Crozier *et al.*, 1980). Accuracy of analysis can be improved using a selective detector, as it responds to the compounds of interest to a much greater degree than it does to the majority of the contaminants (Crozier *et al.*, 1980). Figure 1.5 shows a comparison of U.V absorbance and fluorometric detection selectivity. Unlike U.V. absorption, which is measured as a reduction in light intensity, fluorescence is recorded against a dark background; the resultant improved signal/noise ratio renders fluorescence potentially more sensitive (Sandberg *et al.*, 1987a). The detection limit with a fluorescence detector is usually superior to that of a UV. detector (Figure 1.5). As little as 1 pg of IAA can be detected using reverse-phase HPLC with a fluorescence monitor (Crozier *et al.* 1980). Fluorescence detectors have been widely used for the quantification of endogenous IAA (Crozier *et al.*, 1980; Blakesley *et al.*, 1991b; Prinsen *et al.*, 1992; Garcia-Gomez *et al.*, 1994).

1.14.3 Electrochemical detectors.

The applicability of electrochemical detectors is almost exclusive to reverse-phase and ion-exchange HPLC, as the mobile phase must be electrically conductive (Sandberg *et al.*, 1987a). The limit of detection for IAA is in the order of 50 pg (Sweetser and Swartzfager 1978)

1.14.4 Radioactivity monitors.

Continuous-flow monitoring is desirable if HPLC is to be used in the analysis of radioactive compounds, as collecting and analysing large numbers of small fractions is time consuming. Continuous-flow monitoring of β-radiation in HPLC effluents uses a scintillation technique and, depending on the method of presentation of the eluate to the scintillator, can be classified as either a heterogeneous or a homogenous counting system (Sandberg *et al.*, 1987a). In the homogenous counting system the column eluate is mixed with a liquid scintillation cocktail before passing through a flow cell positioned between the photomultiplier tubes of a liquid scintillation counter. In heterogeneous systems the column eluate moves directly to the flow cell which is packed with a finely divided solid. Lack of sensitivity is one reason why this technique is considered unsuitable for widespread application, however improvements can be made and much depends on chromatographic conditions. Limits of detection of $^{14}C$ isotope are 800 dpm and 200 dpm for heterogeneous and homogenous counting modes respectively (Sandberg *et al.*, 1987a). A recent example where HPLC-radiocounting (HPLC-RC) was employed is with metabolism of IAA by tomato pericarp discs (Catala *et al.*, 1992). They operated in the homogenous mode and also employed the use of HPLC-mass spectrometry as described by Ostin *et al.*, (1992).

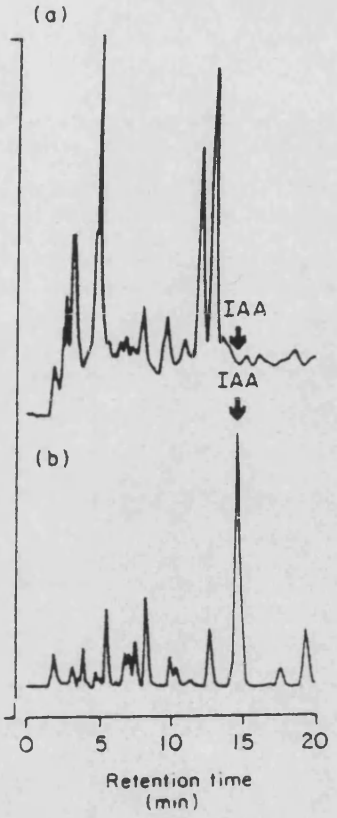



Figure 1.5 Comparison of detector selectivity in an HPLC analysis of IAA from germinating seeds of *Dalbergia dolichopetala*. Detectors (a) absorbance monitor at 280nm, (b) fluorimeter, excitation 280nm, emission 350nm. Column: 250x5mm i.d. 5μm ODS-Hypersil. Mobile phase: 35% methanol in 1% aqueous acetic acid, samples: equal sized aliquots (Sandberg *et al.*, 1987a).

## 1.15 2-methylindolo-α-pyrone assay.

IAA reacts with acetic anhydride to from 2-methylindolo-α-pyrone, (2-MIP), (Plieninger *et al.*, 1964). Although the sensitivity of the 2-MIP assay is influenced by a number of factors including the purity of the reagents and type of spectrofluorimeter used, subnanogram limits of detection can be routinely achieved (Sandberg *et al.*, 1987a). The assay is specific (Stoessl and Venis

1970) and the only two indoles in plant tissues likely to produce fluorescence indistinguishable from that of IAA after derivatisation (4-chloro-IAA and 5-hydroxy-IAA) can be separated prior to or after derivatisation (Lino *et al.*, 1980; Sjut 1981; Blakesley *et al.*, 1983). This assay has been used by several groups of workers (Eliasson *et al.*, 1976; Bottger *et al.*, 1978; Mousdale *et al.*, 1978; Blakesley *et al.*, 1991b).

## 1.16 Immunoassays.

The appeal of immunoassays is based on their low limits of detection, the fact that they require little sophisticated equipment and their potential for processing large numbers of samples (Sandberg *et al.*, 1987a).

### 1.16.1 Radioimmunoassays.

With radioimmunoassay (RIA) in addition to antiserum, an appropriate radiolabelled compound must be available for use as an immunotracer. $^{3}H$-labelled immunotracers are used most frequently due to their high specific activity, $^{14}C$ (Weiler *et al.*, 1981) and $^{125}I$ (Weiler 1981) have also been used, but these have limitations when compared to $^{3}H$ (Sandberg *et al.*, 1987). A RIA for the detection of as little as 0.5-1 pmol of IAA in unpurified or partially purified extracts was reported (Weiler 1981), however, although cross-reactivity was checked, there was no reported check for validity by a suitable technique such as GC-MS. However, the idea of employing immunoassays for accurate quantification with minimal purification has been questioned (Sandberg *et al.*, 1985; Cohen *et al.*, 1987).

### 1.16.2 Enzyme-linked immunoabsorbent assays.

With enzyme-linked immunoabsorbent assay (ELISA) the tracer is labelled with an enzyme, rather than a radioisotope. The antibodies are bound to the walls of either plastic tubes or wells in polystyrene plates. The sample is

added and there is competition between the tracer and hormone present for the available antibody binding sites. After a suitable incubation period the tubes/wells are washed to remove the soluble contents prior to determining the amount of immunotracer bound to the antibodies on the wall, by incubating with an suitable substrate. The amount of product formed from the substrate is measured colourmetrically and is proportional to the amount of bound enzyme labelled tracer which is inversely proportional to the amount of hormone in the sample (Sandberg *et al.*, 1987a). ELISA is more sensitive than corresponding RIAs for IAA because with RIA, the detection of the tracer is based on the relatively few disintergrations of the radiolabelled immunotracer. With ELISA, bound enzyme-labelled tracer can catalyse the conversion of relatively large amounts of substrate to product, thus providing an inherent amplification factor which has the potential to increase the sensitivity of the assay. Sub-fmol sensitivity for measurement of abscisic acid (ABA) in stomatal guard cells using ELISA was reported by Harris and Outlaw (1990). Cohen *et al.*, (1987) compared commercial ELISA kits for quantitative accuracy by GC-MS-SIM. Purification similar to that obtained by at least one high HPLC step was generally necessary prior to ELISA analysis of plant materials. In no case was it possible to obtain reasonable estimates of IAA from crude extracts or even from solvent partitioned fractions of plant tissues. Label *et al.* (1988,1989) used ELISA to quantify endogenous levels of IAA and ABA in *Prunus avium* explants, the purification system included $C_{18}$ Sep-Pak and HPLC. Levels of IAA, ABA, zeatin and zeatin riboside during the course of ARF of *in vitro Sequoiadendron giganteum* were quantified by ELISA (Berthon *et al.*, 1989).

## 1.17 Gas chromatography.

Most indoles are not sufficiently volatile for direct GC and have to be derivatised to increase their vapour pressure prior to analysis.

Gas chromatography detectors:

1.17.1 Flame ionisation detectors.

Flame ionisation detection (FID) was used in the early studies to analyse IAA (Stowe and Schilke 1964; Davis *et al.*, 1968; Grunwald and Lockard 1970). FID permits nanogram levels of IAA derivatives to be quantified. The system lacks sensitivity as almost all organic compounds elicit a response, thus necessitating extensive sample purification for accurate analysis (Sandberg *et al.*, 1987a).

1.17.2 Electron-capture detectors.

An electron capture detector (ECD) offers both sensitive and selective monitoring of components containing highly electronegative groups (Sandberg *et al.*, 1987). With the exception of chlorinated compounds, indoles do not possess such properties and can only be detected after conversion to halogenated derivatives. Bittner and Even-Chen (1975) analysed IAA as chlorinated analogues of IAA-Me and IAA-Et, while Epstein and Cohen (1981) have utilised pentafluorbenzyl esters. Although these derivatives permit picogram levels of detection, selectivity is low when endogenous IAA is analysed as all the carboxylic acid in an extract will be derivatised and aquire electron-capturing properties (Sandberg *et al.*, 1987a). Epstein and Cohen (1991) tried a post-derivatisation $C_{18}$-HPLC step (Figure 1.6a,b). An alternative method is to methylate samples and then derivatise the indole nitrogen with trifluoroacetic anhydride (Brook *et al.*, 1967), heptafluorobutyric anhydride (Bertilsson and Palmer 1972) or pentafluoropropionic anhydride (Lachno *et al.*, 1982). In premethylated samples this approach provides a more selective detection of indoles as only the compounds containing amide moiety

will be derivatised (Sandberg *et al.*, 1987a). The limit of detection is about 10 pg for 1-trifluroacetyl-IAA-Me (TFA-IAA-Me), and 1-heptafluorobutyryl-IAA-Me (HFB-IAA-Me), (Seeley and Powell 1974). Using 1-pentafluoropropionyl-IAA trifluoroethyl ester (PFP-IAA-TFA) analysed by GC-ECD the detection limit is lowered to about 3 pg, however this is at the expense of selectivity when working with plant extracts (Lancho *et al.*, 1982).

1.17.3 Alkali-flame and nitrogen-phosphorus detectors.

Selective detection of IAA following GC is much easier with an alkali-flame detector (AFID) than with an ECD (Sandberg *et al.*, 1987a). Using GC-AFID Schwartz and Powell (1979) analysed IAA in semipurified methylated extracts of immature strawberry fruit and apple shoot tips, and reported that the limit of detection was about 150 pg. An AFID is basically a FID which has an alkali source in contact with the hydrogen flame. However two problems of AFID are firstly that it is very sensitive to fluctuations in the rate of flow of hydrogen. Secondly it has a relatively short life due to rapid evaporation of the metal salt from the source (Sandberg *et al.*, 1987a).

The problems of AFID were overcome with the introduction of the alkali flameless or nitrogen-phosphorus detector (NPD) (Kolb and Bischoff 1974). The background noise of a NPD is much lower and a lot more stable than that of a AFID, and NPD has a much better longevity than an AFID (Olah *et al.*, 1979). GC-NPD has been used to quantify IAA in methylated extracts from *Zea mays* kernels (Martin *et al.*, 1980) and seedlings (Cohen and Schulze 1981) and *Salix pentandra* shoots (Jensen *et al.*, 1986). The detection limit using GC-NPD is around 5 pg (Martin *et al.*, 1980).

1.17.4 Radioactivity detectors.

Like HPLC-RC, GC-radioactivity counting (GC-RC) can be used for metabolism studies (Simpson 1968). GC-RC has only found very limited application as HPLC-RC is technically less complex and offers more sensitive on-line detection of radioactive components (Sandberg *et al.*, 1987a).

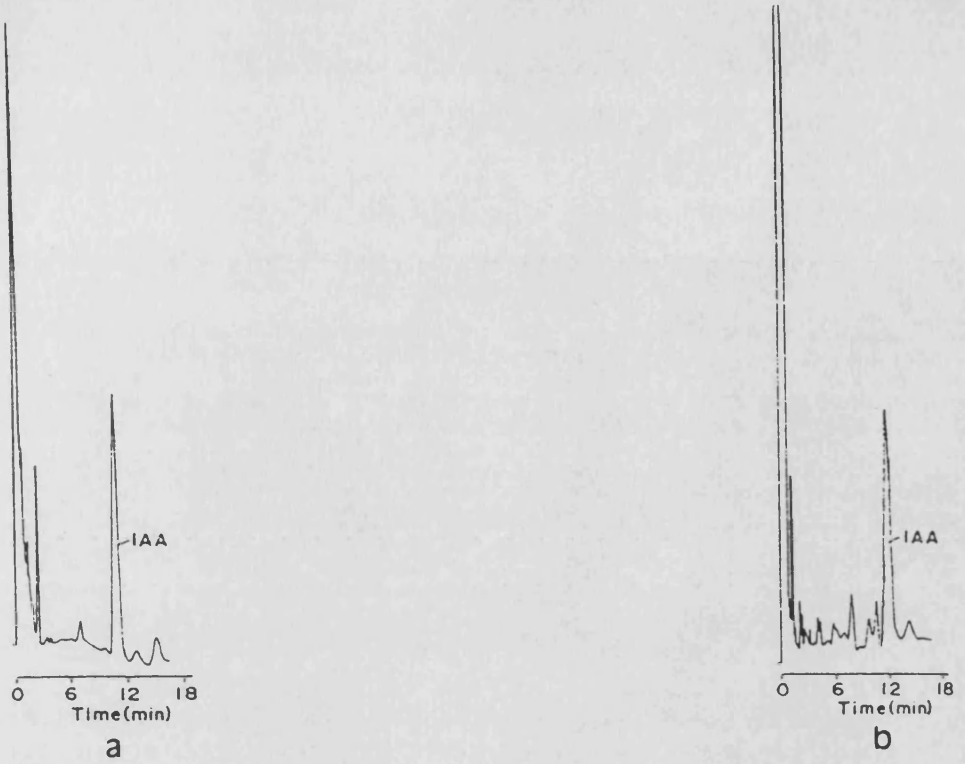



Figure 1.6 a,b. (Epstein and Cohen 1981).

a) GC-ECD of a purified sample from olive leaves using a post derivatisation $C_{18}$-HPLC step. Based on external standardisation, peak represents 133 pg of IAA injected as its pentafluorobenzyl bromide (PFB) ester.

b) GC-ECD of a purified sample, injected without post derivatisation purification. Peak represents 119 pg of IAA injected as its PFB ester.

## 1.18 Gas chromatography-mass spectrometry.

When a gas chromatography column outlet is coupled to a mass spectrometer it becomes a very powerful and flexible analytical tool, enabling mass spectra to be obtained as individual components elute from the gas chromatograph. Computerised data acquisition allows storage and processing of the mass spectra, and enormous amounts of information may be obtained about the chemical nature of complex plant extracts. Improved microprocessor technology has resulted in a new generation of comparatively low cost, bench-

top GC-MS instruments becoming available (Rivier 1986). One of the major limitations of GC-MS as a tool for plant hormone analysis has been the limited availibility of suitable stable isotope labelled 'heavy' internal standards such as $^{13}C_6$-IAA (Cohen *et al.* 1986). All plant hormones, except ethylene must be first converted to volatile derivatives before GC. Generally, carboxylic acids are converted to methyl esters by diazomethane (Figure 1.7)

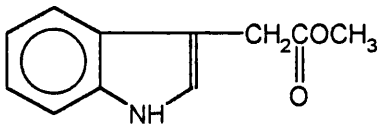



Figure 1.7. IAA-methyl ester (IAA-ME)

In the source, which is maintained under a vacuum of approximately $10^{-6}$ torr, molecules are ionised and fragmented in a beam of high energy electrons. This procedure is known as electron impact (EI) ionisation. The analytical power of mass spectrometry is that fragment ions can be resolved according to their mass to charge ratio (m/z) by means of a magnetic sector or quadrouple mass analyser to give positive-ion spectra which are processed by a computer. Since each mass spectra represents a characteristic fingerprint, identification can be obtained by comparison with reference spectra (Figure 1.8). The presence of one of these spectra in the GC-MS run of a suitably derivatized plant extract and at the correct retention time is conclusive proof of existence in the extract.

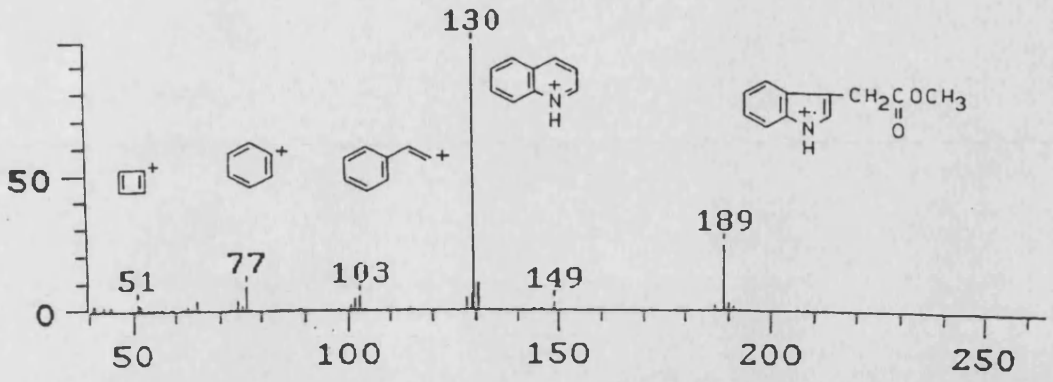
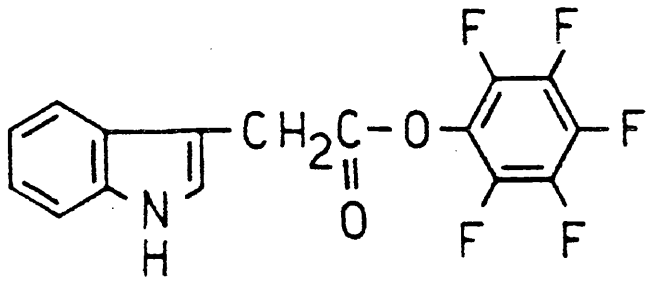



Figure 1.8 EI-GC-MS mass spectrum of methyl ester derivatised authentic IAA standard. (Blakesley, unpublished data).

With IAA-ME the most abundant ion when run under EI bombardment is the quinolinium ion (130m/z) which produces a base peak of 100% intensity and the molecular ion (189 m/z), (Sandberg *et al.* 1987a). The $^{13}C_6$-IAA internal standard with six $^{13}C$ atoms labelling the benzene ring, results in a shift of six units producing corresponding quinolinium and molecular ions at 136 and 195 m/z respectively (Cohen *et al.*, 1986). Since mass spectrometers distinguish between isotopically labelled molecules, GC-MS can be used to measure the relative proportions of an endogenous compound and its heavy isotope internal standard. With quantitative analysis greater sensitivity is achieved when only a few selected ions associated with molecular fragments unique to the hormone of interest are monitored (Dunlap and Guinn 1989). This mode of GC-MS operation is selective ion monitoring (SIM). With IAA the 130 and 189m/z ions, and the 136 and 195 ions from the $^{13}C_6$ internal standard are the most frequently monitored.

Chemical ionisation (CI) involves the chemical reaction of the sample molecules with reagent ions (positive or negative) produced from an appropriate reactant gas in a high pressure ion. Pentafluorobenzyl bromide is a frequently used derivatizing agent (Figure 1.9).




IAA-PFB

Figure 1.9 IAA-pentafluorobenzyl (PFB) ester.

CI is more sensitive than electron impact, five to ten fold ion responses can be achieved with methane positive ion or ammonia negative CI, compared to the EI mode of fragmentation (Rivier and Saugy 1986). Negative ion CI is also a more selective process and has a lower background noise than either positive CI or EI. Therefore even if the intensity of the signal is not much greater, there is likely to be a 20- to 50-fold increase in sensitivity (Sandberg *et al.*, 1987a). Best results are obtained when the compound of interest is naturally electron capturing, for example abscisic acid, (Netting *et al.*,1988). Negative-ion spectra tend not to be used frequently as they generally contain fewer fragments and as a result are less informative for qualitative analysis. Another reason for the rather infrequent use is that the GC-MS equipment necessary for CI is considerably more expensive, although bench-top GC-MS machines with CI capability are now available. One of the only reports utilising negative-ion GC-MS-CI for IAA analysis is by Noiton *et al.*, (1992b), who looked at the effects of serial subculture *in vitro* on the endogenous levels

of IAA and ABA and the rootability in microcuttings of 'Johnothan' apple (Figure 1.10).

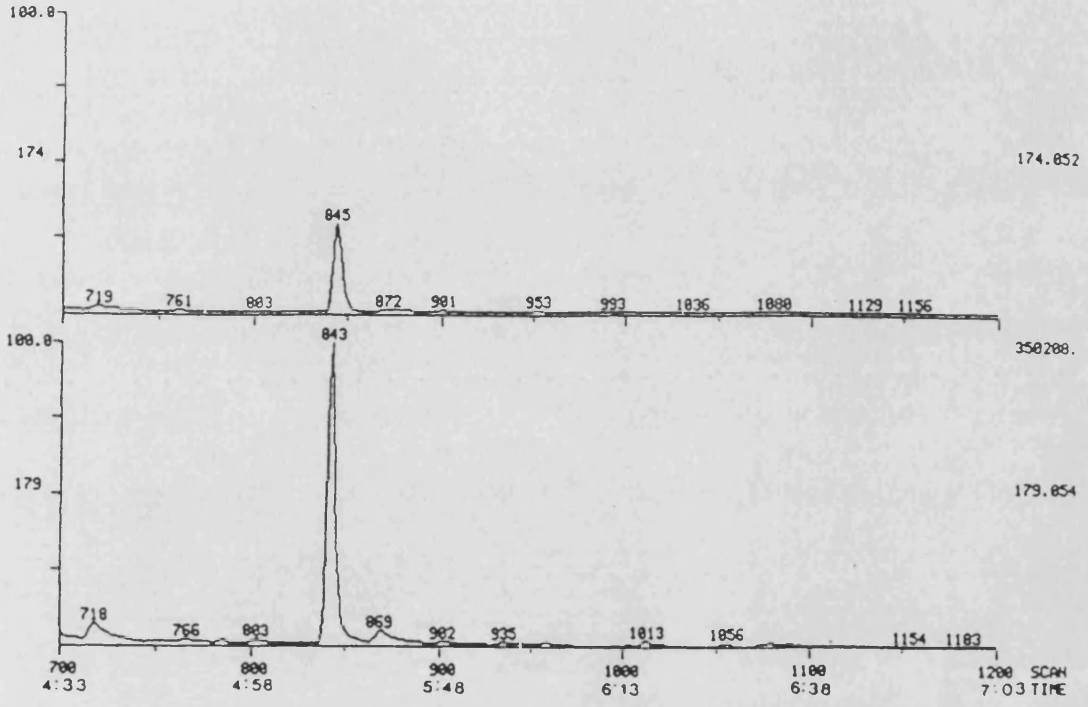



Figure 1.10 The ion chromatographs of m/z 174 (IAA-PFB) ester from extract of 'Johnothan' apple shoots grown *in vitro*. [$^2H_3$] IAA (m/z 179) was added as internal standard. (From Noiton *et al.*, 1992b).

## 1.19 Objectives of the work.

*E. globulus* was chosen for several reasons which include, its economical importance and the interest shown by the CASE awarding body, Advanced Technologies (Cambridge) Ltd. In addition there are dimorphic characteristics between 'old' and 'young' material and the reports of a sharp decline in rooting ability of cuttings with increasing stock plant age.

The primary focus of the work in this thesis is to develop a suitable system which permits accurate quantification of endogenous IAA concentrations in *E. globulus* cuttings. IAA is widely accepted as playing a central role in the rooting process. Quantification of IAA concentration will be predominantly in young 'easy-to-root' seedling cuttings. 'The easy-to-root' material will function as a model system, during which the various stages of ARF will be linked with endogenous IAA concentration. Timing will be crucial if an accurate link between the anatomical events of ARF and IAA concentrations is to be made. Time permitting, IAA concentrations will be measured in older, 'difficult-to-root' cuttings in order to observe whether, or not, IAA concentration varies between the young and old cutting types.

# Chapter 2. Materials and Methods.

## 2.1 Plant material.

Seeds of *Eucalyptus globulus* (Euc 163, "South East Victoria" provenance) and *Eucalyptus grandis* (Euc 180), supplied by Advanced Technologies (Cambridge) Ltd., were stored at room temperature in the dark.

2.1.2 Conditions for glasshouse material.

Seed material for glasshouse propagation was sown in either Fisons No. 1 compost or in vermiculite, contained in seedtrays placed on a propagation bench at 27°C for 5 days before being transferred to glasshouse conditions. The glasshouse had a 16 hour photoperiod with a day and night temperature of 25°C and 18 °C respectively. Seedlings to be grown for more than two months, were potted up in a 3:1:1 mixture of Fisons C.3 compost, coarse bark and perlite respectively, with Ficote F70 (Fisons, 14-14-14 slow-release fertiliser) incorporated into this medium at a rate of 0.3% w/v.

2.1.3 Root initiation.

Rooting trials were carried out on seedlings of different ages in several environments:

(i) Rooting in water: distilled water was contained in 97mm magentas (Sigma, UK) the cuttings were struck with a sharp razor blade, the bases of the cutting were placed through holes in polystyrene floats which suspended them in the water.

(ii) Rooting in a controlled environment cabinet: Cuttings were placed into medium grade vermiculite contained in half-size seed trays with vented plastic lids (Stewart, UK) to help minimise water-stress. Lighting was continuous and the temperature maintained at 25°C in a controlled environment cabinet (Saxcil, UK).

(iii) Rooting on the mist-bench: The mist-bench (Mac Pennie, UK) operated with a dual contact system and was maintained with a base temperature of 27°C, and a 16 hour photoperiod. Auxin treated cuttings were dipped in a 1,000 μM IBA solution (prepared by disolving the IBA in a minimal amount of alcohol and then making to volume with distilled water) for five seconds before being placed in a 50:50 mixture of fine and course sand contained in seedtrays or Plant-Pak P50 units.

(iv) Rooting in the fog-bench: Two days prior to, and immediately after striking cuttings, Rovral (active ingredient iprodine) was sprayed at half strength (0.08% w/v) to help prevent fungal infection. The bases of the cuttings were dipped in a 1,000 μM IBA solution for five seconds and then placed in a 50:50 mixture of fine and coarse sand. The fog-bench base temperature was 27°C and the fogging was intermittent. The fog-bench, based at Advanced Technologies (Cambridge) Ltd., was purpose-built. The fogging setup consisted of a single fog chamber supplied by a single, computer-controlled, fogging nozzel unit which had a ultra violet treated water supply.

## 2.2 Procedures for *in vitro* material.

### 2.2.1 Media preparation.

Murashige & Skoog (1962) medium (MS) (Sigma, U.K.) was used for growing *in vitro* material. In the preparation of media, stock solutions of hormones were added to obtain the desired concentration. Sucrose (BDH), was added to the required concentration and agar (Oxoid, technical grade 0.8% w/v) or phytagel (Sigma, 0.15% w/v) used for solidification. The media were made up to volume and the pH adjusted to pH 5.7 with 0.1M or 1M NaOH or HCL. After dissolving on a hot plate with a magnetic stirrer the media was dispensed into magentas or 75 ml screw cap glass jars using an electric pump and sterilised by autoclaving for 15 minutes at 121°C, 15 Psi.

2.2.2 Sterilisation of plant material.

(i) Seeds. Seeds of *E. globulus* were sterilised by shaking in 20% Co-op bleach (active ingredient sodium hypochlorite) with 2 drops of tween 20 per 100 ml for two 30 minute periods. Following an initial rinse in sterile distilled water, the seeds were washed for two 30 minute periods in sterile distilled water.

(ii) Nodal explants. Explants consisting of 1 or 2 nodes were cut from glasshouse material and sterilised as follows:

Single node explants: shaking for two 3 minute periods in 6% bleach.

Double node explants shaking for two 7 minute periods in 7% bleach.

After sterilisation explants were shaken in sterile distilled water for 20 minutes, then placed in media containing 0.35% w/v activated charcoal (AC, Sigma), 0.03M sucrose and half strength MS. Explants were transferred after 1 week to shoot multiplication medium of semi-solid MS medium supplemented with 0.06M sucrose and 0.75 μM BAP.

2.2.3 Culture procedures and conditions.

The cultures were incubated at 25°C with a 16 hour photoperiod, of light intensity 40 μmol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR), provided by white fluorescent tubing, unless otherwise stated. Subculturing of shoot clumps was carried out at 4-weekly intervals, by separating large clumps into smaller clumps of a few shoots.

2.2.4 *In vitro* rooting

Rooting media for *in vitro* material was either hormone-free or supplemented with IBA.

(A) Hormone-free: 1/4 strength semi-solid MS (1/4 strength macro and micro-elements), supplemented with 0.03 M sucrose.

(B) Incorporation of IBA: a two stage process was utilised.

(i) Root initiation medium: 1/4 strength semi-solid MS, supplemented with 10 μM IBA, 0.03 M sucrose. This media was used for the first 3-7 days, the aim of which was to encourage the early phases, i.e. root induction and early initiation. After this the explants were transferred to a root elongation medium.

(ii) Root elongation media: semi-solid MS, supplemented with 0.03 w/v AC and 0.03 M sucrose.

## 2.3 Histology.

### 2.3.1 Fixation.

Material used in the rooting trials was severed with a sharp razor blade and immersed immediately in formalin acetic acid (O' Brien and Mc Cully, 1981) for a minimum of 48 hours. Glasshouse grown material was fixed in 70% FAA, tissue culture grown material in 50% FAA.

### 2.3.2 Dehydration.

Following fixation tissue was dehydrated by passing through a series of ethanol and 2- methyl propane-2-ol (TBA) solutions (Table 2.1).

Table 2.1 Dehydration of material.

| Stage | Time (hours). |
|---|---|
| 1. 50% ethanol | 2 |
| 2. TBA 1* | 2 |
| 3. TBA 2 | 2 |
| 4. TBA 3 | 2 |
| 5. TBA 4 | 2 |
| 6. TBA 5 | 2 |
| 7. TBA 6 | 12 |

Key: * Refer to appendix (A.7) for the TBA dilution series.

2.3.3 Infiltration.

Dehydrated tissue was infiltrated with wax (Paramat, BDH). The wax beads were added a few at a time every 4 hours for the first 12 hours in an oven at 60°C. Over the next 12 hours approximately 10 beads every 4 hours were added. TBA was evaporated off by removing the tops of the glass vials and leaving them in the oven for 12 hours. The wax was then changed for fresh molten wax twice at 12 hourly intervals.

2.3.4 Embedding.

Rectangular boats, constructed from card, were filled with molten wax and material was transferred to the wax with two pairs of fine tweezers.

2.3.5 Sectioning.

Blocks were prepared by trimming with a sharp razor blade. Sections 7 μm thick were cut on a Reichert microtome with a Cresson-original lung steel blade. The ribbons were expanded by floating on warm water (approximately 50°C), and transferred with a fine paintbrush to microscope slides smeared with Haupt's solution (Appendix A.6). Slides were left to dry on heating blocks at 20°C.

2.3.6 Staining.

Dried slides were loaded onto carrier trays and taken through the rehydration/staining (safranin/fast-green)/dehydration procedure (Table 2.2), for material where the root primordia are likely to be organised toluidine blue was used (Table 2.3).

Table 2.2 Procedure for staining sections with safranin/fast-green.

| Solution | Time (min.) |
|---|---|
| Four passes in 100% histology | 3 each |
| 50/50 histology/100% ethanol | 3 |
| 100% ethanol | 3 |
| 95% ethanol | 3 |
| 80% ethanol | 3 |
| 70% ethanol | 3 |
| 50% ethanol | 3 |
| 30% ethanol | 3 |
| Distilled water | 3 |
| 1% safranin in distilled water | 45 |
| Three passes in distilled water | 1 each |
| 30% ethanol | 1 |
| 50% ethanol | 0.5 |
| 70% ethanol | 0.5 |
| 80% ethanol | 0.5 |
| 95% ethanol | 0.5 |
| 0.5% fast-green in ethanol | 0.5 |
| Two passes in 95% ethanol | 0.5 each |
| 50:50 histoclear/100% ethanol | 1 |
| Five passes in 100% histoclear | 10, or more, each. |

Table 2.3 Procedure for staining sections with toluidine blue.

| Solution | Time (min.) |
|---|---|
| Four passes in 100% histoclear | 3 each |
| 50:50 histoclear/ 100% ethanol | 3 |
| 100% ethanol | 3 |
| 95% ethanol | 3 |
| 80% ethanol | 3 |
| 70% ethanol | 3 |
| 50% ethanol | 3 |
| 30% ethanol | 3 |
| Distilled water | 3 |
| 1% toluidine blue in distilled water | 1 |
| Two passes distilled water | 1 each |
| 30% ethanol | 0.5 |
| 50% ethanol | 0.5 |
| 70% ethanol | 0.5 |
| 80% ethanol | 0.5 |
| 95% ethanol | 0.5 |
| 100% ethanol | 1 |
| 50:50 histoclear/100% ethanol | 1 |
| Five passes histoclear | 10, or more, each |

## 2.4 Analysis of endogenous IAA.

### 2.4.1 Extraction. (Methods from D. Blakesley, Personal Communication).

For initial work, large amounts of woody stem and / or seedling shoot material was used for the extraction, purification and analysis of endogenous IAA. Subsequently for the development of techniques with higher sensitivity levels, smaller quantities of plant material in the extraction stage were necessary.

i) For large, > 1g fresh weight (fwt.), quantities of shoot material: the samples were frozen immediately in liquid nitrogen and then freeze-dried. The freeze-dried samples were stored in a freezer at -70°C until extraction. A mill (Janke & Kunkel) was used to grind the material in a few drops of 80% methanol (MeOH, Rathburn Chemicals Ltd., HPLC grade) containing 0.025% w/v butylated hydroxytoluene (Sigma). The samples were then placed in 30 mls of 80% MeOH, and an internal standard, 100-250 ng of $^{13}C_6$-IAA (kindly donated by Dr. Peter Hedden, IACR, Long Ashton, UK) was added at this stage together with approximately 30,000 dpm 2-$^{14}$C IAA ( 2.04 GBq mmol/l Amersham) radiotracer, and left to stir in a cold room (4°C) overnight.

ii) For small (< 1g fwt.) samples, the plant material was frozen in liquid nitrogen, freeze-dried and then ground in a mortar and pestle containing a few mls of liquid nitrogen. Ten mls of 80% MeOH was used to take up the sample, 1-50ng of $^{13}C_6$-IAA was added and the suspension was left to stir in a cold room overnight. The amount of $^{13}C_6$-IAA added to the samples depends to a large extent on the mass and type of plant sample being extracted. As it is the ratio of $^{13}C_6$-IAA to IAA that is used to calculate the amount of IAA, providing the characteristic ions from both $^{13}C_6$-IAA and IAA can be measured the initial level of $^{13}C_6$-IAA added is not all that important.

2.4.2 Purification for IAA analysis.

The quantity and type of plant material being used dictated which purification steps were incorporated in the purification procedure. The various steps which were used are presented in figure 2.1.

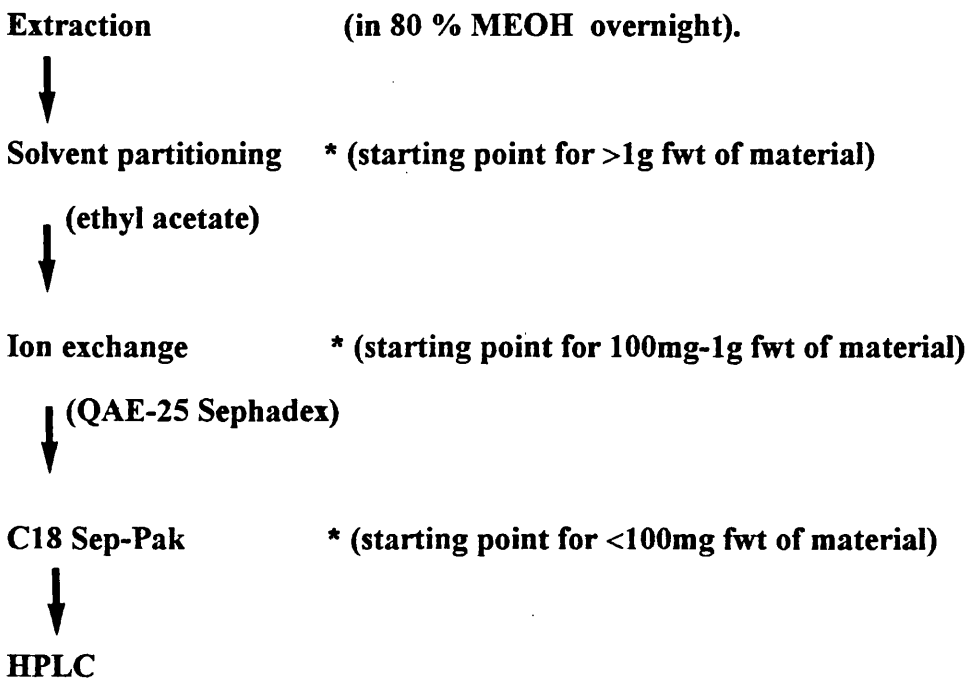
(i) Solvent partitioning. (Methods from D. Blakesley *et al.*, 1991b).

Samples were reduced to the aqueous phase by rotary evaporating at 40°C under vacuum and taken up in 30 mls of 0.5M $K_2HPO_4$ buffer (pH 8) (analar grade, BDH). Following this each sample was washed 3 times in ethyl acetate (analar grade, BDH). The pH was then adjusted to 3.5 using HCl and the sample extracted 3 times into ethyl acetate reduced to dryness by rotary evaporation and taken up in 3mls of pH 8 water.

(ii) Ion exchange chromatography. (Methods from D. Blakesley, Personal Communication).

Sephadex QAE-25 (Sigma) in bond-elute cartridges (Waters Associates) was used for an ion-exchange step. Two-three mls of MeOH followed by pH 8 water was used to moisten the filter. One cm depth of Sephadex, (Appendix A.5, preparation of Sephadex), was poured into the cartridge and conditioned with 4 column volumes of pH 8 water. The sample, which was adjusted to pH 8 using KOH, was carefully loaded onto the column using a fine pipette, then washed with 4 column volumes of pH 8 water. After attaching a conditioned Sep-Pak cartridge (running through 5mls of MeOH followed by 10 mls of pH 3 water) to the base of the bond-elute cartridge, the sample was eluted onto the Sep-Pak cartridge in 10 mls of 7% formic acid (analar, BDH).

**Figure 2.1 Purification protocol for the purification of *E. globulus* tissue for analysis of endogenous IAA.**



(iii) C-18 Sep-Pak. (Methods adapted from Sandberg *et al.*, 1987b)

After loading the sample, the cartridge was washed with 10 mls of pH 3 water. For samples over 100mg fwt. a wash with 10 ml of 10% MeOH was incorporated after the initial wash in pH 3 water to remove more contaminating substances. IAA was eluted in 5 mls of 50% MeOH, dried in a speed-vac to complete dryness and stored dry at -70°C in a freezer until required for HPLC.

(iv) HPLC. (Methods adapted from D. Blakesley *et al.*, 1991b).

Samples in 25 µl 40 % MeOH were injected onto an HPLC (Gilson, 305 pumps, with a UV detector, model 116). The flow rate was 1ml/minute, a C-18 reverse phase 250mm by 4.6mm 5µm column (HPLC Technology, UK) and a C-18, 5µm guard column (Anachem) were employed, detection was at 280nm. Water in the reservoir was reduced to pH 3 by the addition of acetic acid ('HyperSolv' grade, BDH). The HPLC program was isocratic at 40% MeOH for 15 mins (to elute the IAA) followed by a gradient over two mins to 100 % MeOH where it was held for 20 mins (to clean the column). Following HPLC, samples were dried in a speed-vac to complete dryness and stored dry at -70°C in a freezer until derivatization.

2.4.3 Derivatisation.

Two methods of sample derivatisation were employed prior to analysis by GC-MS. Diazomethane was synthesised (Schlenk and Gellerman, 1960), and used to methylate samples analysed by electron-impact GC-MS, and pentafluorobenzyl bromide (PFB, Sigma) was used for chemical-ionisation GC-MS.

i) Derivatisation using diazomethane (Method from Rivier and Saugy, 1986) - for positive-ion electron-impact GC-MS.

100 µl of MeOH was added to the dried sample then an excess of diazomethane, added to methylate the sample. 15 mins later samples were dried under a stream of nitrogen.

ii) Derivatisation using PFB (Method from Epstein and Cohen 1981) -for negative-ion chemical-ionisation GC-MS.

50 µl of acetone was added to the dried sample followed by 1 µl of N-ethyl piperidine (Sigma). 5 µl of PFB (Sigma) was added, screw tops were placed on the 1 ml glass vials which were kept at 60°C for 45 mins after which the samples were dried under a stream of nitrogen.

2.4.4 Gas chromatography-mass spectrometry. (Methods from P. Hedden and Mervin Lewis, Personal Communication).

(i) Electron impact positive-ion GC-MS (using a bench-top set-up).

The electron energy was 70 eV, and the carrier gas used was helium. The injection port was held at 220°C,and the transfer line at 250°C. Samples were injected on to a BP5 column (SGE, 0.25μM film thickness) in the split-splitless mode. The GC oven was kept at 60°C for 2 minutes after sample injection, then heated to 200°C at 20°C/ minute, and to 300°C at 4°C where it remained isothermal.

(ii) Electron impact positive-ion GC-MS (using a magnetic sector GC-MS)

Samples were analysed using a Kraytos MS80 RFA GC-MS. Electron energy was 70 eV, and the carrier gas used was helium. The source was maintained at 200°C and the interface at 260°C. Samples were injected on a SGE BP10 column (25m x 0.32mm, 0.5μm film thickness) using oncolumn injection.The GC oven was kept at 70°C for 1 minute after sample injection, then heated to 200°C at 15°C/minute, and to 270°C at 4°C/minute where it stayed isothermal. Selected ion monitoring (SIM) was employed at 3,000 resolution, with a dwell time of 0.23 seconds.

(iii) Chemical ionisation negative-ion GC-MS.

When the Kraytos GC-MS was run in chemical-ionisation (CI) mode, PFBta was used as a reference compound for calibration and lockmass (= 200). Methane was used as the carrier gas (pressure 0.5 Bar), and the source was maintained at 180°C. Samples were injected on an OV-1701 SAC column (15m x 0.32mm, 0.5μm film thickness) using the split-splitless mode. The GC oven was kept at 100°C for 1 minute after sample injection, heated to 200°C at

20°C/minute, and then to 280°C at 5°C/minute where it remained isothermal. SIM was carried out at 3,000 resolution, with a dwell time of 0.20 seconds.

2.4.5 Calibration of $^{13}C_6$ IAA on a bench-top GC-MS.

Varying amounts of IAA:$^{13}C_6$ IAA were added to form a calibration curve, (see ratios below).

| Quantity of $^{13}C_6$ IAA (ng) : | IAA (ng) |
|---|---|
| 100 | 0 |
| 100 | 1 |
| 100 | 5 |
| 100 | 10 |
| 100 | 25 |
| 100 | 50 |
| 100 | 100 |
| 100 | 200 |
| 100 | 500 |

The amount ratio: response ratio curve (Fig. 2.2a,b) shows that over a wide range of IAA levels the $^{13}C_6$ IAA was accurately quantified. The calibration data was automatically integrated on the GC-MS using non-linear regression analysis and resulting experimental data applied to it.

2.4.6 Calibration of $^{13}C_6$ IAA on a Kraytos GC-MS.

Calibration of the Kraytos GC-MS involved variable ratios of authentic IAA to $^{13}C_6$-IAA standards being injected on to the columns and the resulting data being input in to Minitab for regression analysis. Subsequent experimental data was calibrated by applying it to the regression analysis carried out on the authentic standards.

Figure 2.2a. Amount ratio/ response ratio curve for IAA (130 m/z / 136 m/z ions).

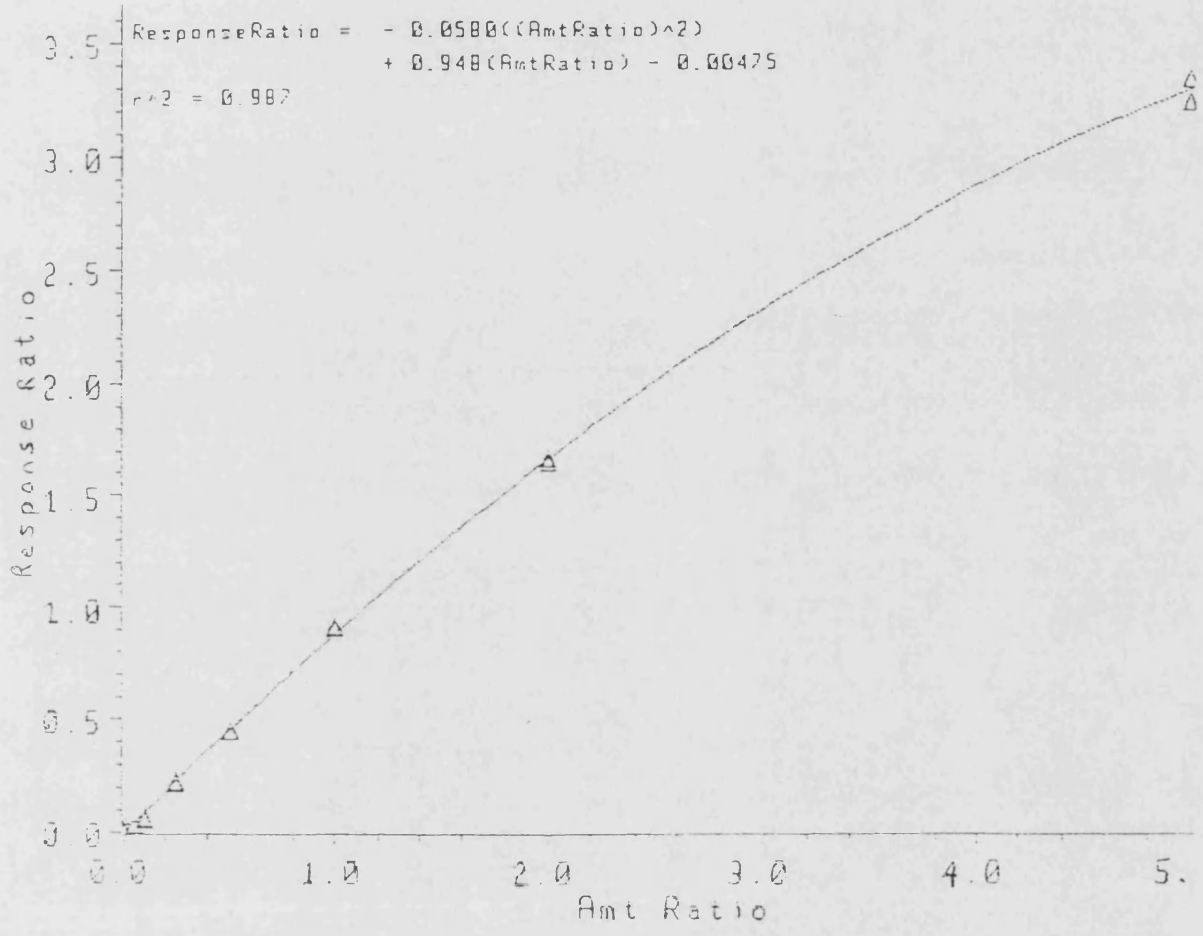
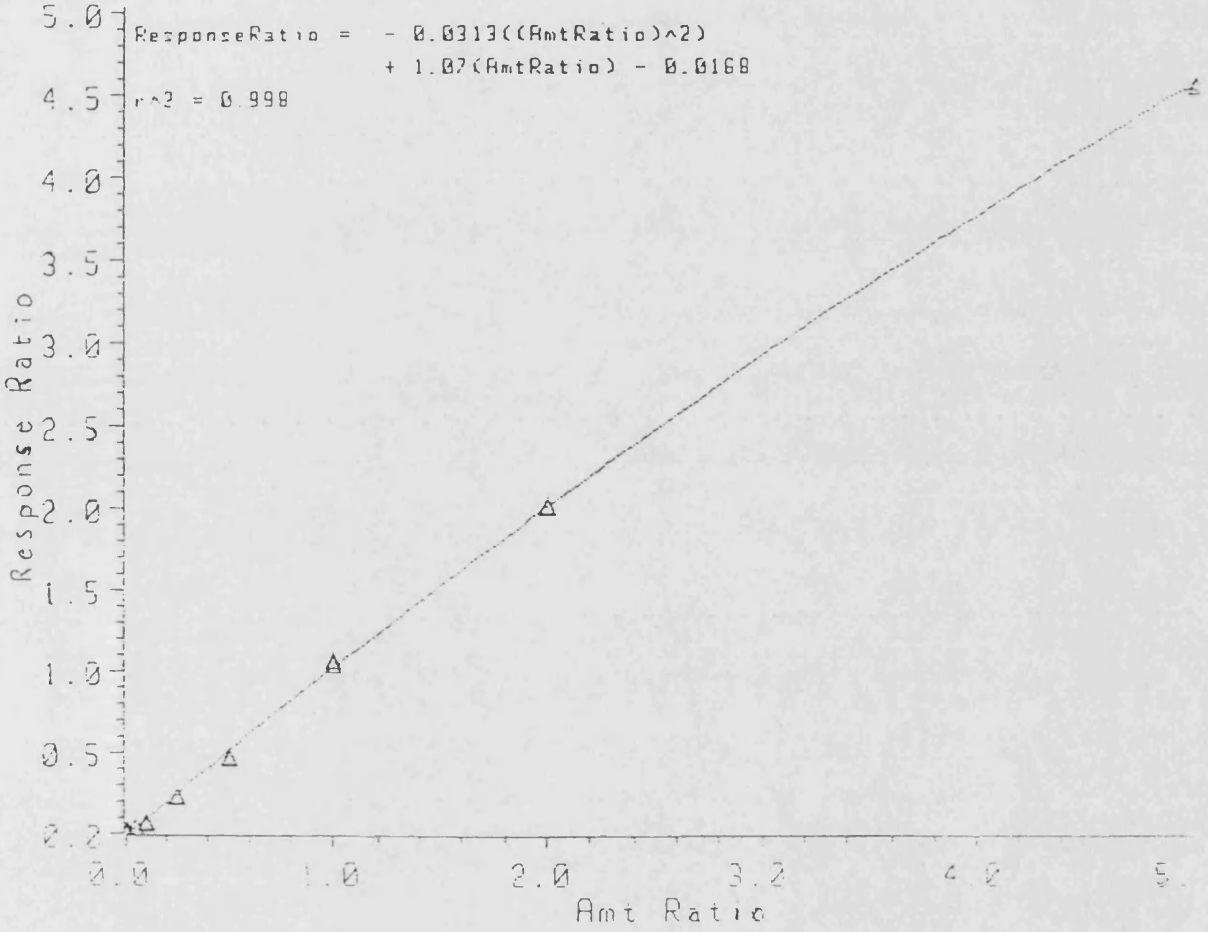


Figure 2.2b Amount ratio/ response ratio curve for IAA (189 m/z / 195 m/z ions).



## 2.5 Statistical analyses.

Where there was a wide range of cutting ages with a consistent decline in the rooting percentage, it was not necessary to perform statistical analysis. In other cases Chi-square was used to test significance levels with rooting percentage where necessary. For concentrations of IAA, the student's t-test was used to test significance levels.

# Chapter 3. Rooting.

## Introduction.

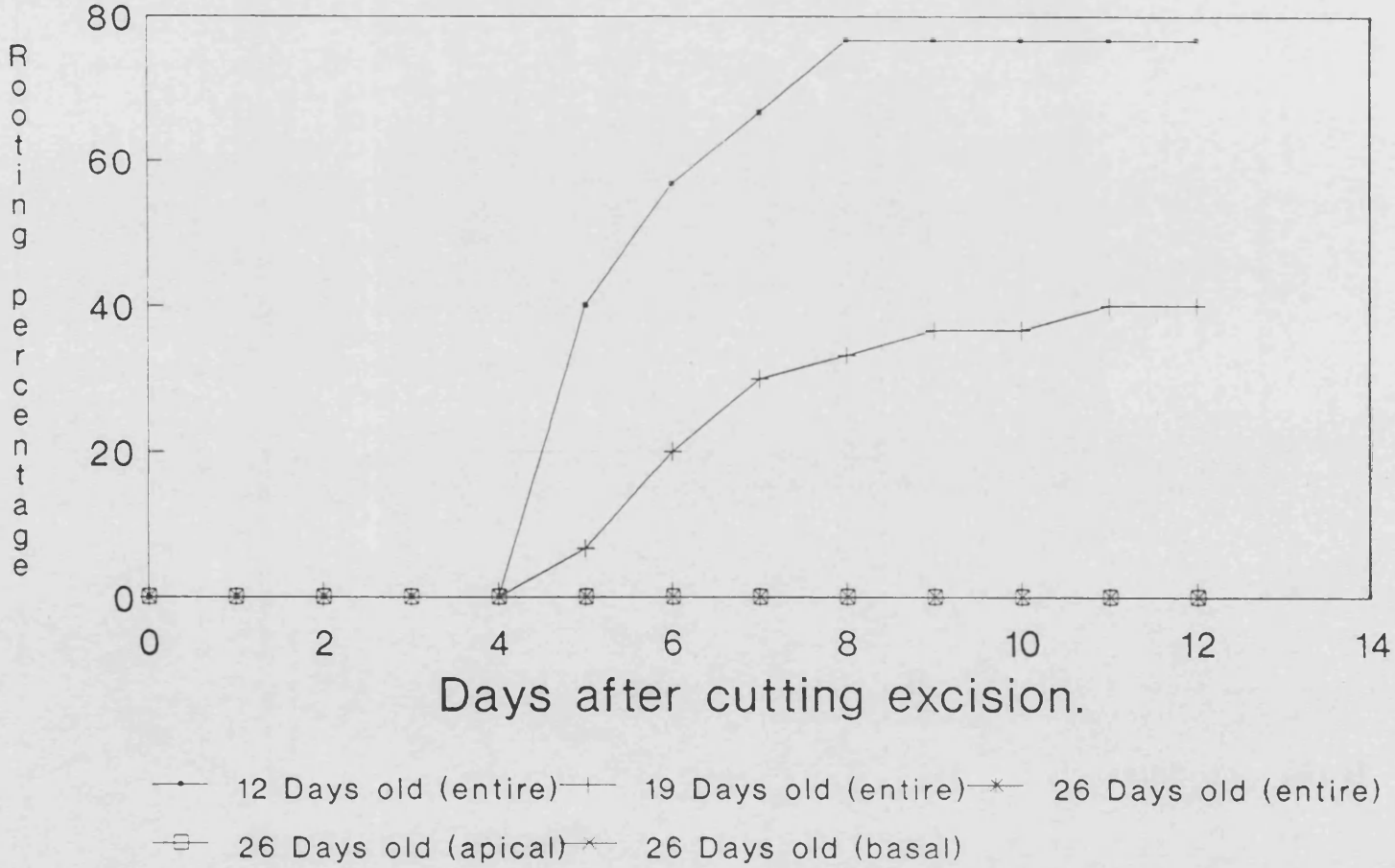
A major problem for many tree improvement programmes is that by the time trees are mature enough to identify elite genotypes, it is often impossible to propagate clonal material. *E. globulus* is a notoriously difficult-to-root species (Hartney, 1982). There is a need with *E. globulus* to study the problem of the decline in rooting ability as stock plant material increases in age and to quantify the decline. The use of seedling material in this work is as a model system. The aim is to identify cuttings of different types and ages suitable for further study, in particular looking for tissue with different rooting abilities in order to see whether or not there is a link between ease of rooting ability and endogenous IAA level.

## 3.1 (a) The rooting in water of glasshouse-grown *Eucalyptus globulus* seedling cuttings of different ages.

The aim of this experiment was to determine the effect of seedling age on the rooting ability of glasshouse-grown *E. globulus* cuttings. Seeds were sown in Fisons F1 compost contained in seedtrays at intervals, to enable cuttings of different ages (12,19 and 26 days old) to be taken on the same day. At 12 days old, the 1st true leaf pair of the seedlings was expanding; at 19 days, the 2nd true leaf pair were expanding and at 26 days the 3rd true leaf pair were expanding and the fourth leaf pair were upright. Cuttings were prepared by severing the shoot system 1 cm below the cotyledons. The growth of 26 day old seedlings, enabled three cutting types to be taken: entire cuttings, prepared by severing the shoot system 1 cm below the cotyledons; apical cuttings consisting of the apical 3 nodes and basal cuttings, consisting of the basal 3 node pairs. The rooting chambers consisted of 97mm magentas with polystyrene floats, with holes through which the bases of cuttings were placed with the basal leaf pair suspended the cuttings above the distilled water. Six replicate magentas were used, each containing 5 cuttings. The magentas were incubated in a growth room with a 16 hour photoperiod at 25°C (Section 2.2.3).

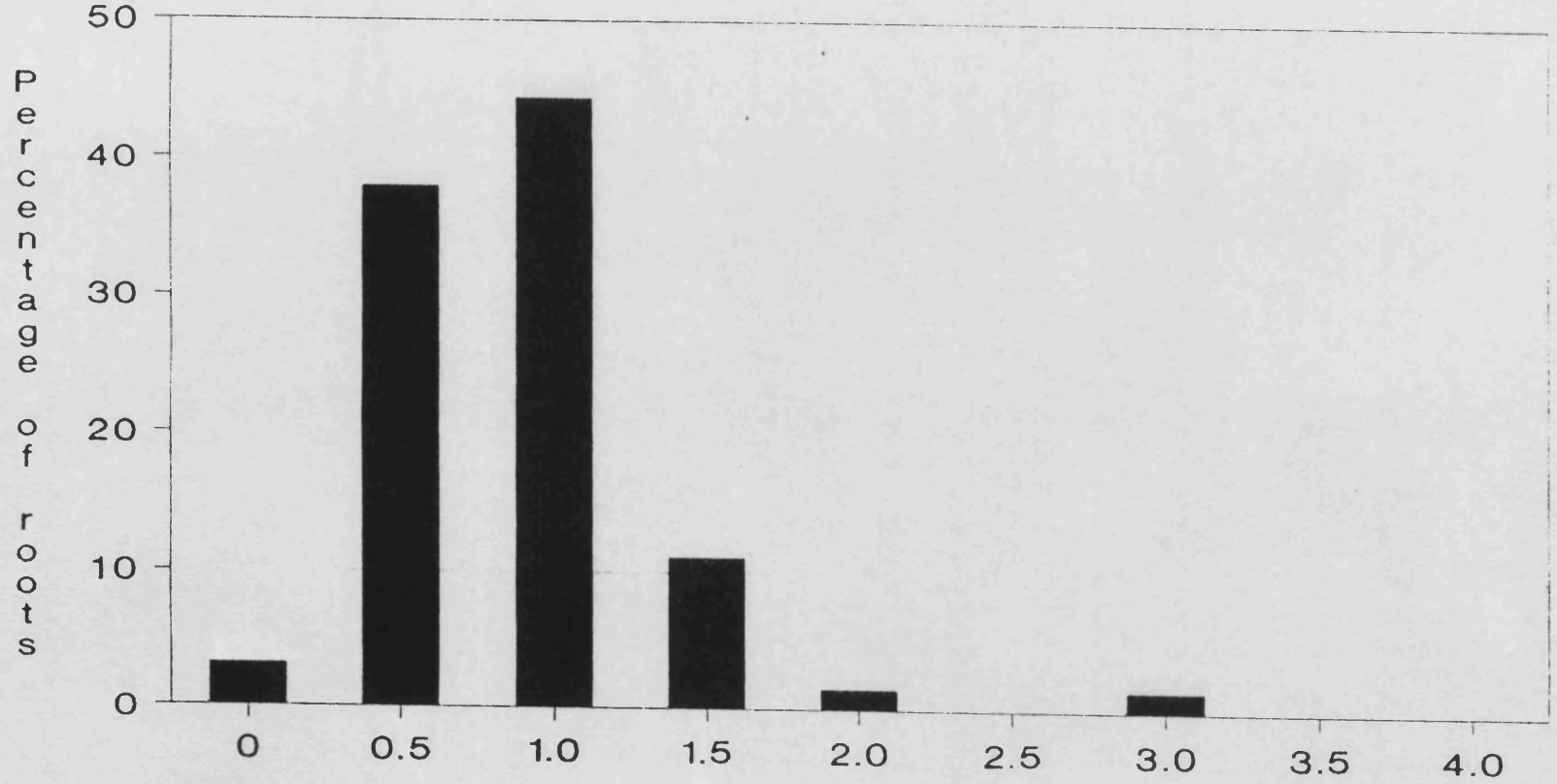
During the first two days after striking the cuttings, the basal few millimetres of hypocotyl curved upwards forming a u-shaped bend. Four days after cutting excision the bases of some of the hypocotyls started swelling and the first roots began to emerge 5 days after cutting excision. New roots continued to be observed up to 8 and 12 days after cutting excision in the 12 and 19 day old cuttings respectively (Figure 3.1). The rooting ability decreased sharply with increasing age of the seedling stock material (Figure 3.1). At 12 days old 77% of the cuttings rooted, whereas the rooting

Figure 3.1 The effect of seedling age on the rooting of *in vivo E. globulus* cuttings.




Key: apical = apical 3 nodes, basal = basal 3 nodes, entire = from the cotyledons upwards.

Figure 3.2 The distribution of roots in 12 day old *in vivo E. globulus* seedling cuttings.




n=30

percentage of 19 day old declined significantly ($x^2$<0.05) (Figure 3.1) to 40%. Cuttings from 26 day old seedlings did not form any roots on the basal or entire cuttings. The mean root number per rooted cutting was 1.32, 1.17, and 0 for the 12, 19 and 26 day old cuttings respectively. Ninety seven percent of all roots emerged within 2mm of the cutting base (Figure 3.2).

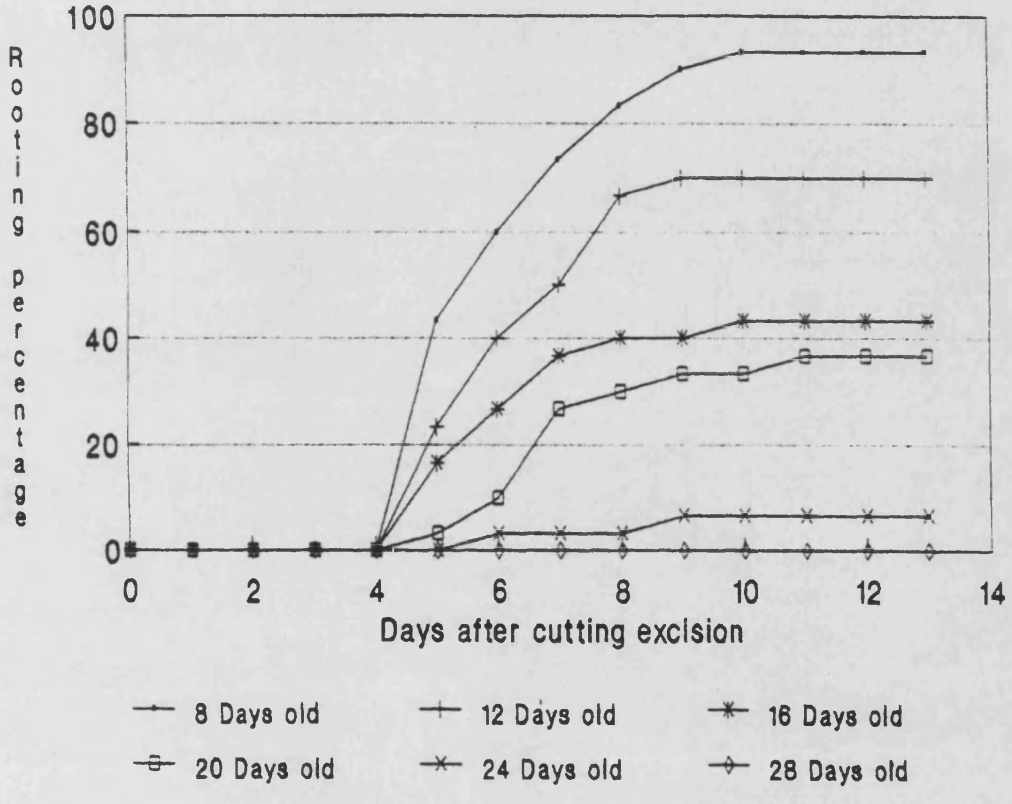
(b) A second experiment was carried out with a wider age range of seedlings using the same experimental design in order to examine more carefully the decline in rooting ability. Cuttings were excised from seedlings of 8,12,16,20,24, and 28 days, the growth stages of which are shown in Table 3.1.

Table 3.1 The growth stage of *in vivo E. globulus* seedlings.

| Seedling age (Days) | Seedling growth stage. |
|---|---|
| 8 | 1st true leaf pair upright. |
| 12 | 1st true leaf pair expanding. |
| 16 | 2nd true leaf pair expanding. |
| 20 | 3rd true leaf pair expanding. |
| 24 | 3rd true leaf pair expanding/expanded |
| 28 | 3 rd true leaf pair expanded. |

The same upward bending in the basal few millimetres of the hypocotyl described in experiment 3.1a was observed here. A rapid decrease in rooting ability with increasing stock plant age was again clearly visible (Figure 3.3). The rooting percentage was high in the 8 and 12 day old material (93% and 73% respectively), but the rooting percentage of 16 day old had declined to 40% (Figure 3.3). There was a further significant ($x^2$<0.05) decline in the rooting ability, from 36.7% to 6.7% between the 20 and the 24 day old material respectively. The 28 day old material did not form roots. The sharp

Figure 3.3 The effect of seedling age on the rooting of *in vivo E. globulus* cuttings.



decline in the percentage of cuttings which formed roots in this experiment was very similar to that seen in experiment 3.1a.

## 3.2a Rooting of different aged *in vitro E. globulus* seedling cuttings.

The aim of this experiment was to investigate the rooting of seedlings germinated *in vitro* and to see how the rooting compared to *in vivo* cuttings. Six sterilised seeds (Section 2.2.2) were sown per magenta, on 1/2 strength MS, supplemented with 0.03 M sucrose and solidified with 0.15% w/v phytagel (Section 2.2.1). One week after germination the seedlings were selected for uniformity and thinned to 4 per magenta. Six replicate magentas, each containing 4 cuttings were used for each harvest. The seedlings were severed 1cm beneath the cotyledons to form the cuttings. Cuttings were excised from seedlings of 12,16,20,24,28,32,36 and 40 days old and placed into the rooting medium to a depth of 1 cm. The rooting medium, contained in magentas was 1/4 strength MS, supplemented with 0.03 M sucrose and solidified with 0.15% w/v phytagel. The growth stage of the *in vitro* seedlings (Table 3.2) was very similar to that of the *in vivo* material, except at 36 days old, where the 4$^{th}$ leaf pair was still expanding in the *in vitro* seedlings, but had already expanded in the *in vivo* seedlings. Cuttings were prepared by severing the seedlings 1cm beneath the cotyledons. A sharp decline in rooting ability with increasing stock plant age was recorded (Table 3.3). Twelve day old seedling cuttings had a rooting ability of 80%, which was similar to that of the *in vivo* system (Figure 3.1). However, in contrast to the *in vivo* system, the subsequent decline in rooting ability with increasing seedling age of the *in vitro* system occurred more gradually. The mean root number per rooted cutting did not decrease significantly with increasing seedling age (Table 3.3).

Table 3.2 The growth stage of *in vitro E. globulus* seedlings.

| Seedling age (Days) | Seedling growth stage |
|---|---|
| 12 | 1st true leaf pair expanding/expanded, 2nd leaf pair upright. |
| 16 | 2nd true leaf pair expanding. |
| 20 | 2nd true leaf pair expanded, 3rd leaf pair upright. |
| 24 | 3rd true leaf pair expanding. |
| 28 | 3rd true leaf pair expanding/expanded, 4th leaf pair upright. |
| 32 | 4th true leaf pair expanding. |
| 36 | 4th true leaf pair expanding. |
| 40 | 4th true leaf pair expanded, fifth leaf pair upright. |

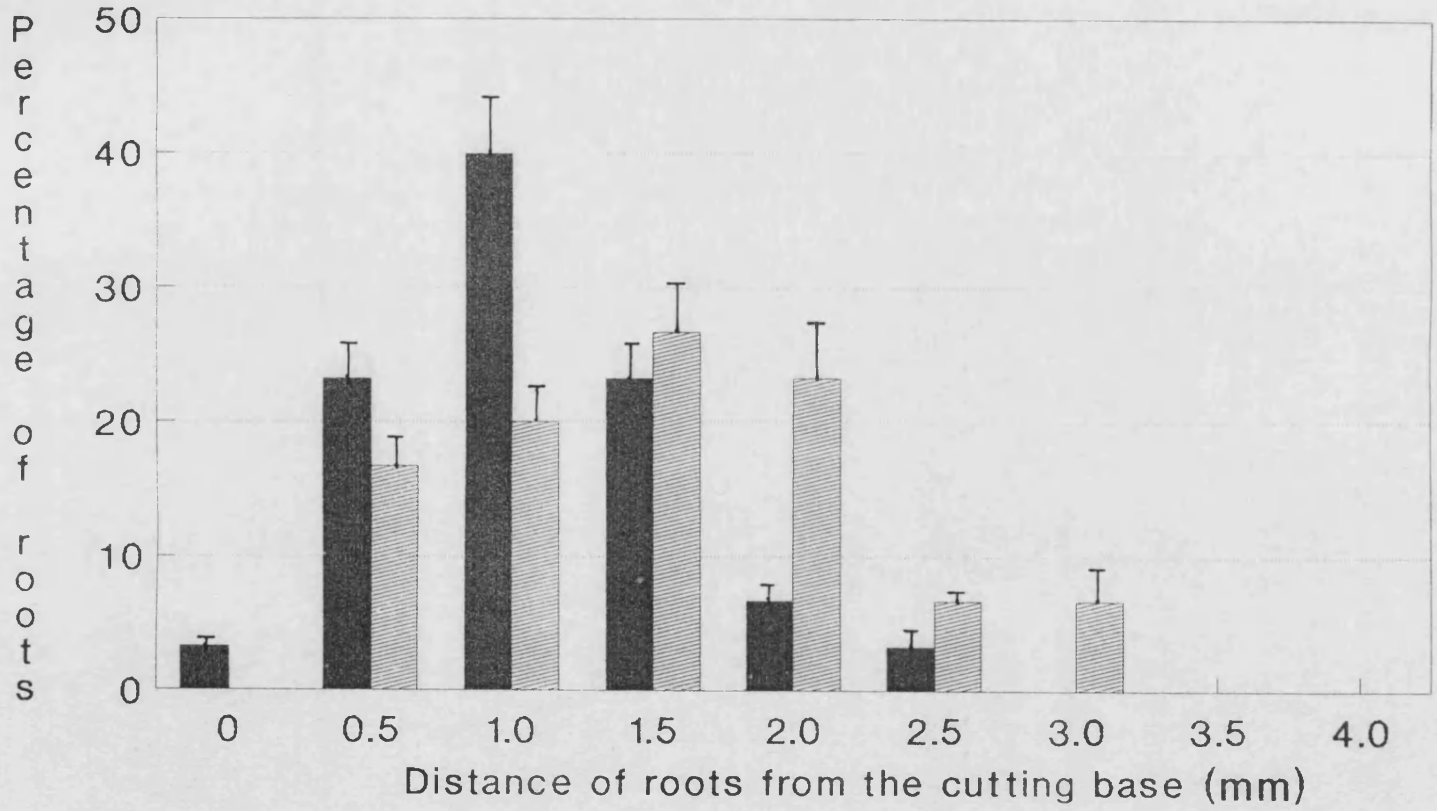
Conditions in the growth room were as described in the materials and methods (Section 2.2.3). The number of rooted cuttings, number of roots per cutting and the distance of the roots from the cutting base was recorded at daily intervals.

Almost without exception the roots appeared within the basal 2mm of hypocotyl (Figure 3.4). There was not a significant difference in the distribution of roots in relation to the cutting base between the 12 and 20 day old material. The experiment showed a very sharp decline over the four day period between the 16 and 20 day old material.

Table 3.3 The effect of seedling age on the rooting of *in vitro E. globulus* cuttings.

| Age of seedlings (Days) | Rooting percentage | Mean root number/rooted cutting |
|---|---|---|
| 12 | 79.2 | 1.5 |
| 16 | 70.8 | 1.6 |
| 20 | 41.6 | 1.5 |
| 24 | 25 | 1.7 |
| 28 | 37.5 | 1.3 |
| 32 | 37.5 | 1.3 |
| 36 | 29.2 | 1.3 |
| 40 | 37.5 | 1.0 |

Figure 3.4 The distribution of roots on *in vitro E. globulus* cuttings.




n=24 ⊤= Standard deviation.

3.2b A second experiment was carried out with a wider range of *in vitro* germinated seedlings. Cuttings were excised from 4,8,12,16,17,18, 19,20,21,28,35,42 and 49 day old *in vitro* seedlings. In addition, cuttings were placed individually into medium contained in boiling tubes to permit 24 replicate containers in contrast to six when magentas were used (Experiment 3.2a).

In boiling tubes the decline in the rooting ability with increasing stock plant age was again evident, but more gradual (Figure 3.5a-c) than that in the previous experiment where magentas were also used as rooting chambers (Table 3.3). In boiling tubes however, the rooting ability remained high for longer, at 21 and 28 days old the rooting ability was 95.3 and 79.17% respectively (Figure 3.5b) whereas in magentas, it was 41.6 and 37.5% for these two ages (Table 3.3); for both ages the rooting percentage was significantly different ($x^2<0.01$) between the two vessel types. The conditions in the two types of container could possibly explain the significant difference in rooting percentage between experiments 3.2a and 3.2b. The stock seedlings were, as far as leaf-pair number and general appearance, the same in both experiments, as were the growth room conditions. As with experiment 3.2a, the mean root number per rooted cutting did not decrease significantly with increasing seedling age (Table 3.4).

Table 3.4 Mean root number per rooted cutting of *in vitro E. globulus* cuttings. (Boiling tubes used as rooting chambers).

| Cutting age (Days) | Mean root number/ rooted cutting. |
|---|---|
| 12 | 1.9 |
| 16 | 1.5 |
| 17 | 1.6 |
| 18 | 1.7 |
| 19 | 1.7 |
| 20 | 1.6 |
| 21 | 1.2 |
| 28 | 1.2 |
| 25 | 1.4 |
| 42 | 1.3 |
| 49 | 1.4 |

Figure 3.5a The effect of seedling age on the rooting of *in vitro E. globulus* cuttings (boiling tubes used as rooting chambers).

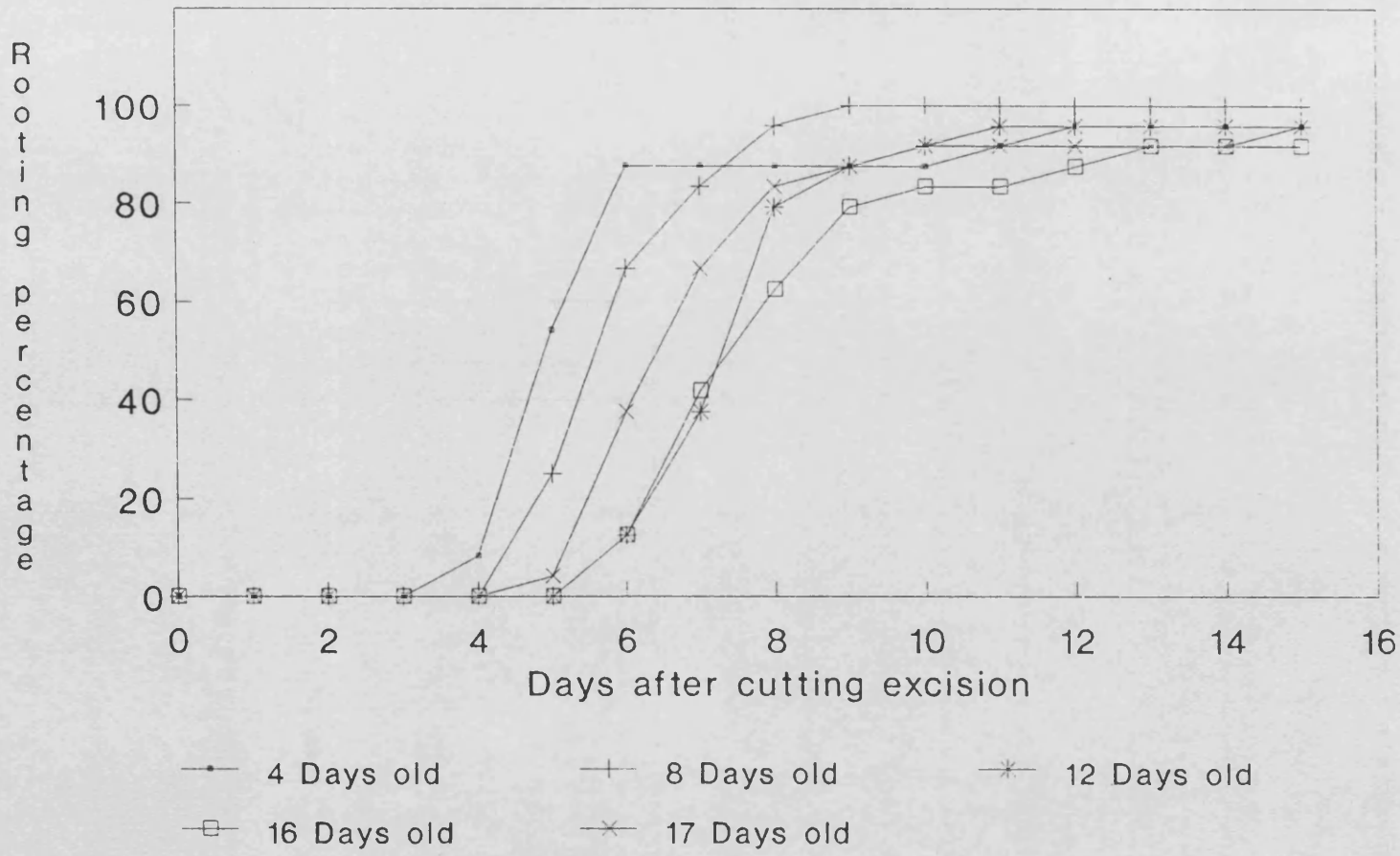


Figure 3.5b The effect of seedling age on the rooting of *in vitro E. globulus* cuttings (boiling tubes used as rooting chambers).

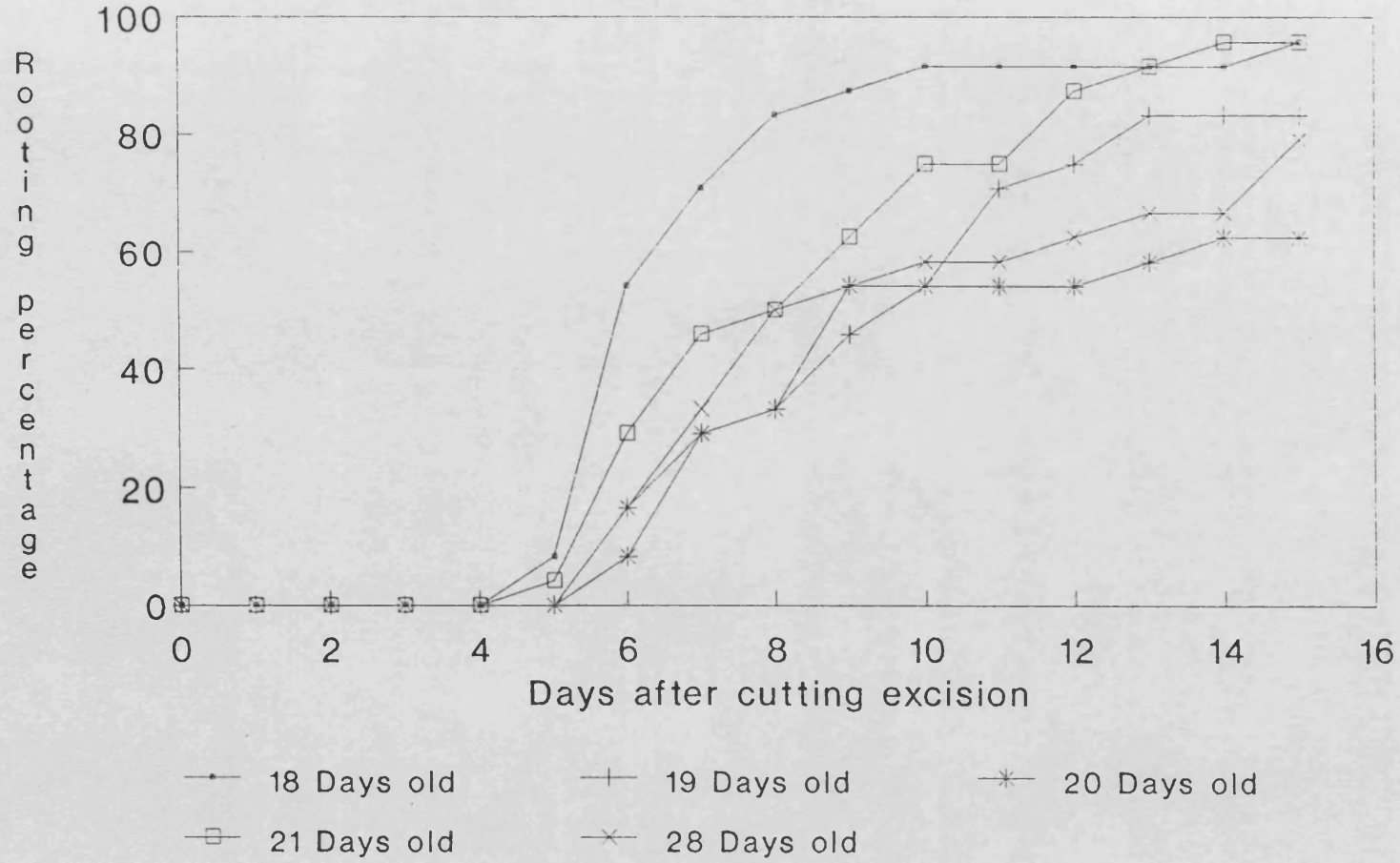
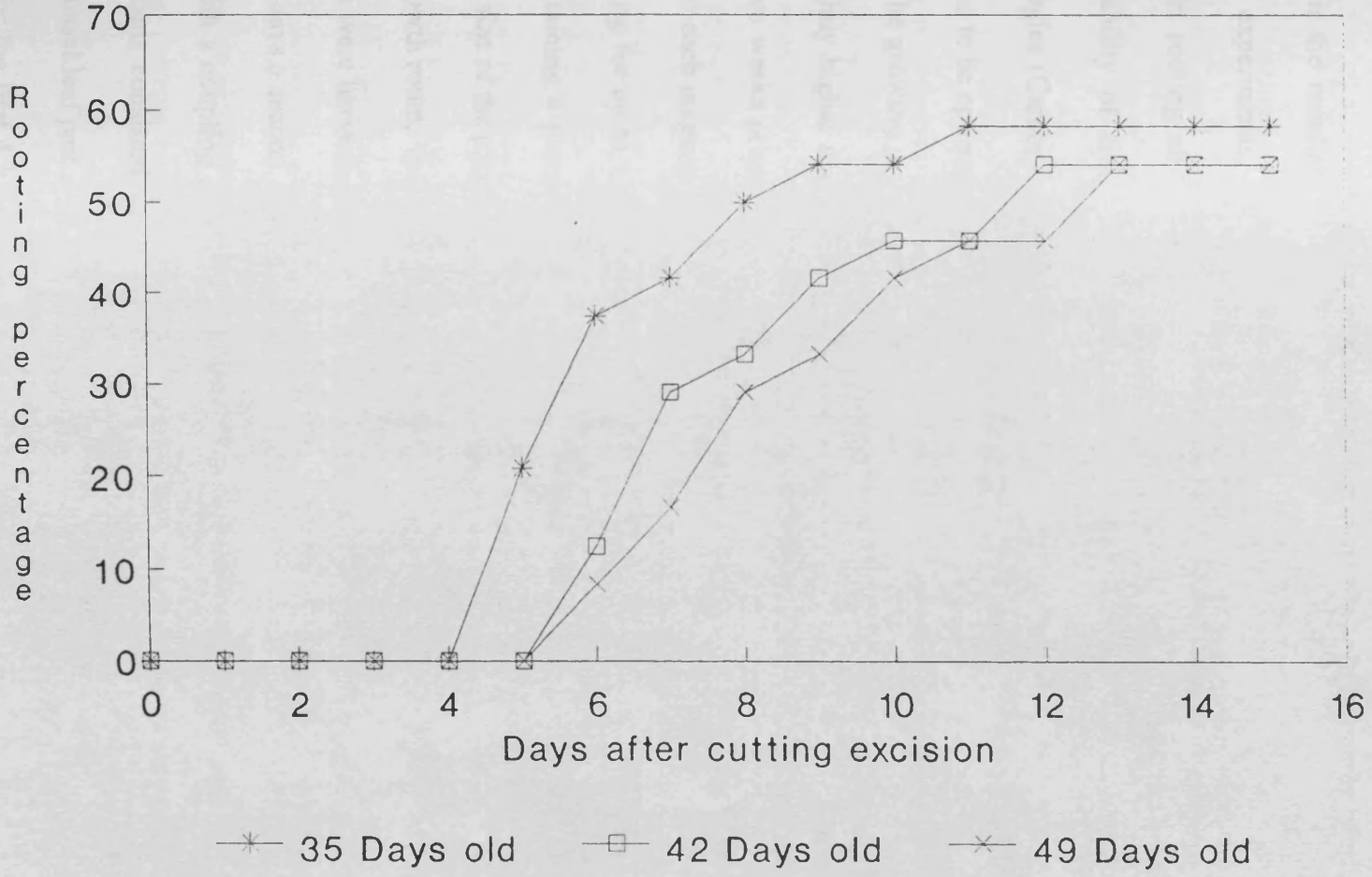


Figure 3.5c The effect of seedling age on the rooting of *in vitro E. globulus* cuttings (boiling tubes used as rooting chambers).



## 3.3 The effect of IBA on the rooting ability of *in vitro E. globulus* seedling cuttings of different ages.

The aim of this experiment was to investigate the effect of IBA on the decline in the number of cuttings that root as stock plants age found in previous experiments. Cuttings from older material were used to see if the decline in rooting ability continues still further. The effect of IBA on the rooting ability of similar *in vitro* explants has been tested by Advanced Technologies (Cambridge) Ltd. (ATC), and a pulse of 10-20 μM for 3-7 days was found to be optimal.

The growing media for the seedlings was essentially as before, although with slightly higher sucrose (0.06 M), and was based on work carried out at ATC. Two weeks prior to each harvest, 20 mls of 1/2 strength liquid MS was applied to each magenta to elevate the nutrient level as the stock plants would be growing for up to 135 days in the same container. Six replicate magentas, each containing 4 plants were used. Magentas were employed as they permit the large size of the plants in this experiment. Due to high contamination levels in the growth room, magentas were sealed with a strip of parafilm. The *in vitro* seedlings were harvested 14, 30, 45, 75, 105 and 135 days after germination. After 30 days a second magenta was inverted and linked on top of the magenta below with a coupling ring, to accommodate the increasing size of the plants. The explants consisted of the vigorously growing apical three node cuttings with the basal leaf pair removed.

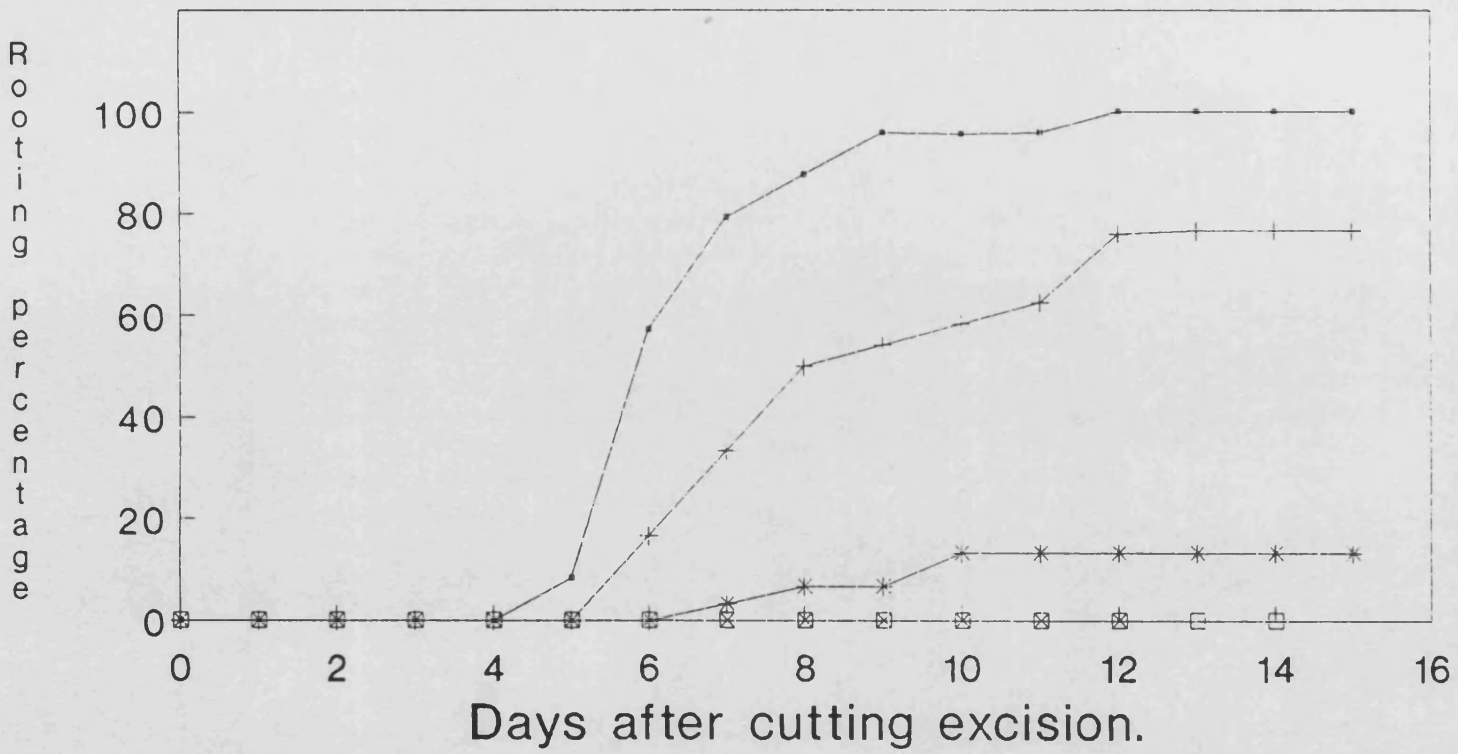
For the first 4 harvests a hormone-free rooting medium was used, this consisted of 1/4 strength MS, 0.03 M sucrose, solidified with 0.15% w/v phytagel. From 75 days old onwards the rooting protocol consisted of a two stage media system:

1) Root initiation media for 5 days: 10 μM IBA, 1/4 strength MS, 0.03 M sucrose, solidified with 0.15% w/v phytagel. Then transfer to:

2) Root elongation media: 0.3% w/v activated charcoal, 1/4 strength MS, 0.03 M sucrose, solidified with 0.15% w/v phytagel.

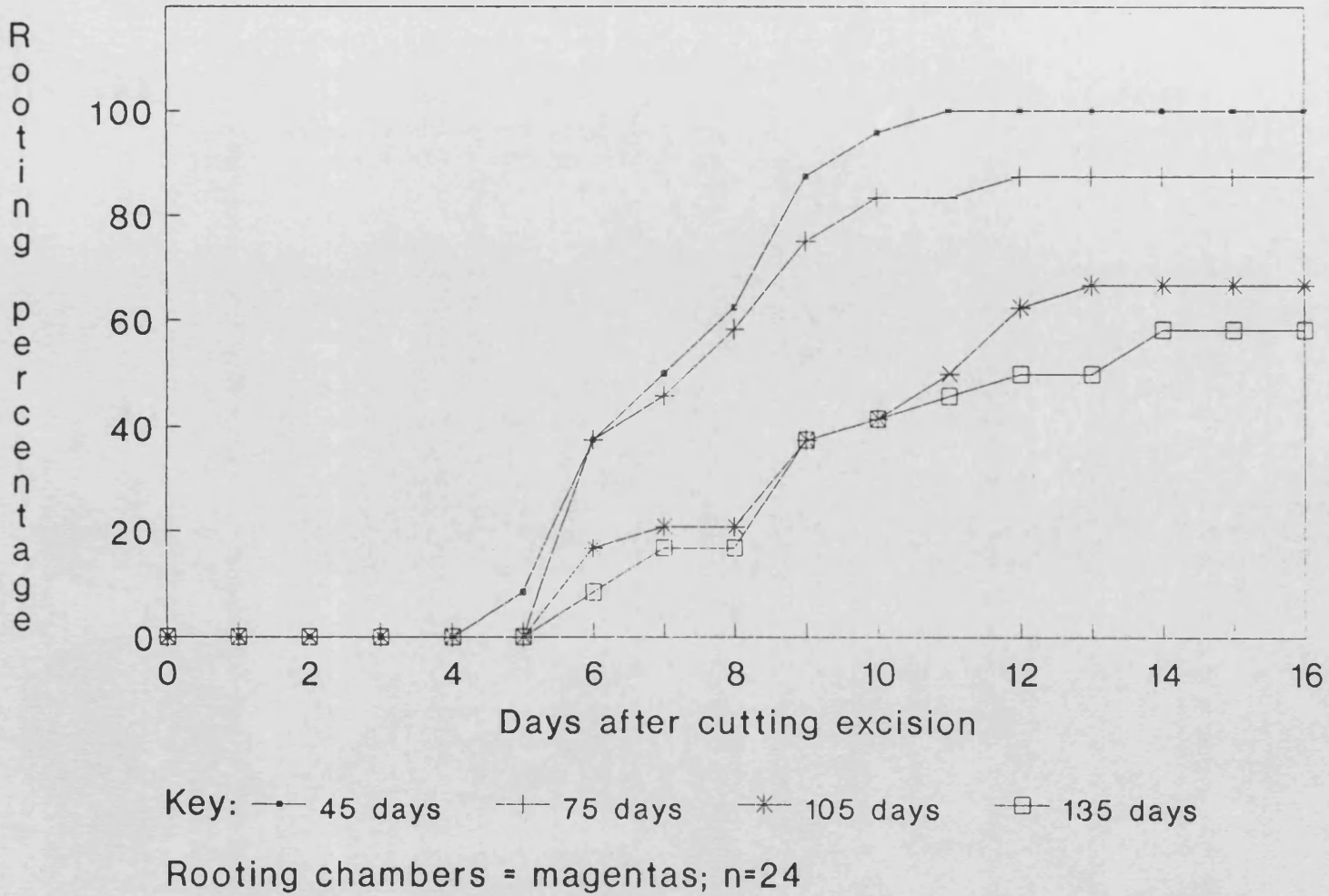
On hormone-free media the rooting ability declined rapidly, from 100% of 14 day old seedlings to only 12% of 45 day old seedlings, and the 75 day old stock material failed to root at all (Figure 3.6a). Also in this experiment the magentas were sealed with parafilm resulting in higher humidity which resulted in poorer growth, for example the occurrence of callus on leaves. The magentas were sealed with parafilm to overcome problems of contamination during this experiment, caused by fluctuating temperatures in the growth room. In experiment 3.2a the rooting percentage of 32-40 day old stock material was between 30 and 40 % (Table 3.3) The incorporation of a 5 day 10μM IBA pulse considerably improved the rooting ability of the material (Figures 3.6a and b). With the IBA pulse 100% of the cuttings from the 45 day old stock material rooted, whereas on the hormone-free medium only 12% of these cuttings formed roots (Figures 3.6a and 3.6b). The rooting ability still declined with age when the cuttings were treated with an IBA pulse, although at a much slower rate. Fifty percent of the 135 day old cuttings formed roots (Figure 3.6b). The time of root emergence also varied with age, after being transferred to root elongation media 100 % of the 45 day old material had rooted within 10 days, whereas 135 day old material took 14 days for all the cuttings that were going to root to do so (Figure 3.6b). The rooting zone was also larger with the IBA pulse, roots emerged within the basal 10mm of the hypocotyl. It would be interesting to increase the age of material being rooted

Figure 3.6a The effect of age on the rooting of *in vitro E. globulus* explants.




Rooting chambers = magentas; n=24

Figure 3.6b The effect of age on the rooting of *in vitro E. globulus* explants incorporating a 10 μM pulse.



to see whether the rooting percentage declined further over subsequent months or stabilised.

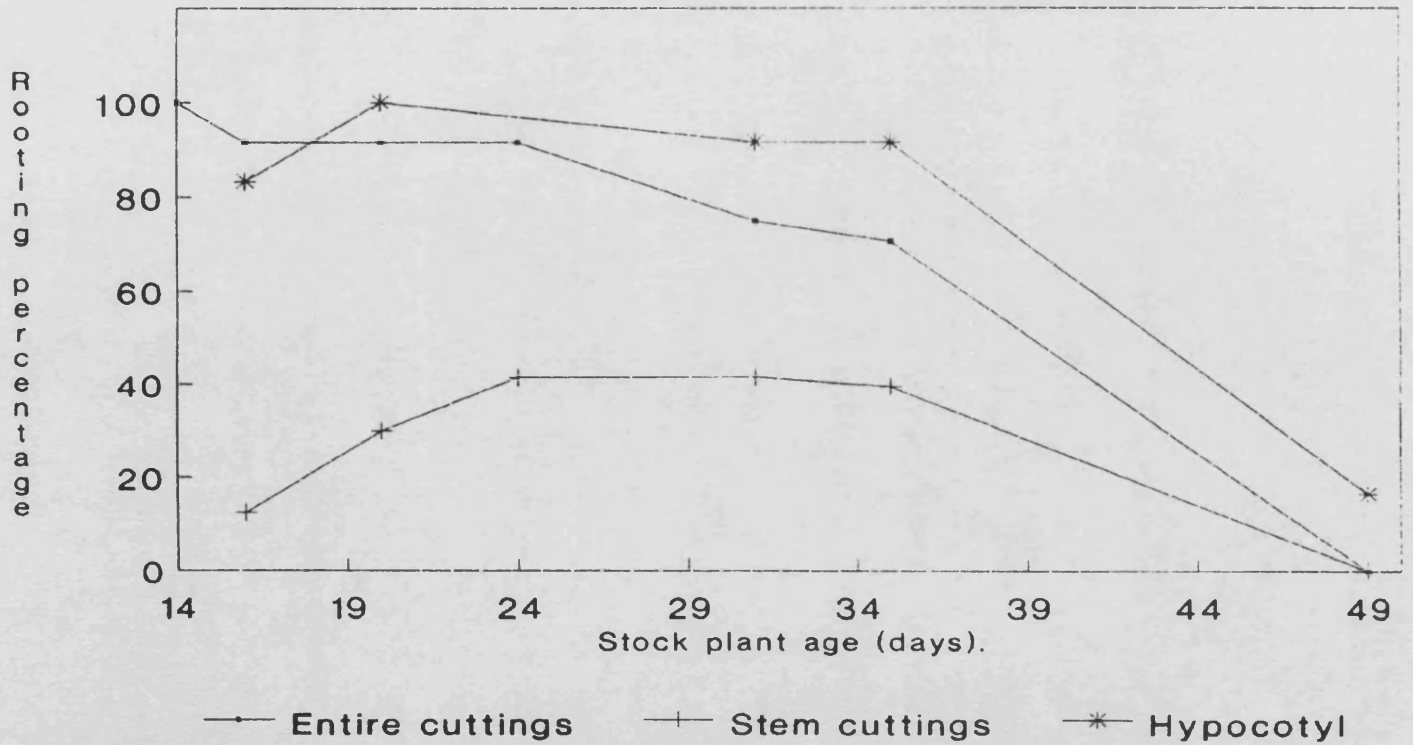
## 3.4 Rooting of *in vitro E. globulus* seedling cuttings.

The aim of this experiment is to record the *in vitro* rooting of cuttings of different ages and to see if the rooting ability varies in different cutting types with increasing stock plant age. Sterilised seeds (Section 2.2.2) were sown in 1/2 strength MS, supplemented with 0.06 M sucrose and solidified with 0.15% w/v phytagel (Section 2.2.1). Parafilm again was used to seal the magentas, which were incubated in a growth room at 25°C with a 16 hour photoperiod (Section 2.2.3). The previous experiment only used 1 type of cutting, which consisted of the vigorously growing apical three leaf pairs with the basal leaf pair removed, in this experiment 3 types of cuttings will be used, entire cuttings consisting of seedlings severed 1cm beneath the cotyledons, stem cuttings seedlings which were severed just above the cotyledons, and hypocotyl cuttings in which seedlings are severed 1cm beneath the cotyledons and just above the cotyledons. (Table 3.2). Six replicate magentas each containing 4 cuttings, were set up for each harvest date. Six replicate magentas each containing 4 cuttings, were set up for each harvest date. Harvesting occurred at the following ages, 14,16,20,24,31, 35 and 49 days after germination. The hormone-free rooting media was 1/4 strength MS supplemented with 0.03 M sucrose and solidified with 0.15% w/v phytagel. In addition, the 49 day old cuttings were given a 10 μM pulse of IBA in the rooting medium. After the 3 day IBA pulse cuttings were transferred to a root-elongation medium which consisted of 1/4 strength MS supplemented with 0.03 M sucrose and solidified with 0.15% w/v phytagel; 0.3% w/v activated charcoal was incorporated to adsorb the residual IBA in order to encourage elongation of root-primordia.

The stem cuttings had a lower rooting ability than either the entire or hypocotyl cuttings (Figure 3.7), except with the 49 day old material where a

10μmol pulse was incorporated (Table 3.5). The decline in rooting ability with increasing stock plant age up to 35 days old was a gradual process in entire and hypocotyl cuttings. With stem cuttings the pattern was quite different; the rooting percentage of 16 days old cuttings was only 12.5% and this increased to 41.6% for the 31 days old cuttings (Figure 3.7). The 49 days old material was also quite different, the stem and entire cuttings did not root at all, and only 16.67% of the hypocotyl cuttings rooted (Figure 3.7). The 10 μM IBA pulse increased the percentage of cuttings which formed roots. The most responsive material were the entire cuttings where the percentage rose from zero on hormone-free media to 75% after the IBA pulse. In this experiment only 18% of the 49 day old hypocotyl cuttings on hormone-free media rooted and the rooting percentage of the entire and stem cuttings was 0% (Figure 3.7). The rooting percentage of 40 day old entire cuttings in experiment 3.2a was almost 40% (Table 3.3), possible explanations for the difference between the two experiments include the use of a different seed batch (but still the same provenance) and that the magentas had to be sealed with parafilm in experiment 3.4 due to high levels of contamination in the growth room. Over 50% of 49 day old cuttings rooted in experiment 3.2b (Figure 3.5c), however boiling tubes were used to contain the cuttings in this experiment and conditions in the rooting environment could be quite different.

Figure 3.7 The effect of stock plant age on the rooting of *in vitro E. globulus* seedling cuttings. (Rooting chambers = magentas).




Entire = seedlings severed 1 cm. beneath the cotyledons; stem = severed just above the cotyledons; hypocotyl= seedlings severed 1 cm. beneath the cotyledons and immediately above the cotyledons. The rooting medium was hormone-free, 1/4 MS, supplemented with 0.03M sucrose and solidified with 0.15 w/v phytagel.

Table 3.5 The rooting percentage of three cutting types from 49 day old *in vitro* seedlings in the presence/absence of IBA in the rooting medium.

| | Media type | |
|---|---|---|
| Cutting type | Hormone-free medium | IBA pulse |
| Entire | 0 | 75 |
| Stem | 0 | 33.3 |
| Hypocotyl | 16.67 | 29.2 |

Key: Hormone-free medium = 1/4 strength MS supplemented with 0.03 M sucrose and solidified with 0.15% w/v phytagel. The IBA pulse was a 10 μM IBA pulse for 3 days after which cuttings were transferred to a root-elongation medium which consisted of 1/4 strength MS supplemented with 0.03 M sucrose and solidified with 0.15% w/v phytagel; 0.3% w/v activated charcoal was incorporated to adsorb the residual IBA in order to encourage elongation of root-primordia.

## 3.5 Rooting ability of glasshouse-grown *E. globulus* and *E. grandis* of different ages.

This experiment was designed to record the rooting percentage of cuttings from different positions on *E. globulus* and *E. grandis* trees grown under glasshouse conditions. It was carried out at ATC early on in this project, to identify suitable cuttings from this type of material which might be incorporated into the study of endogenous IAA levels in cuttings if time permitted. Three ages of stock plants; 16 weeks, 20 weeks and 25 weeks were used for the two species and all plant material was of seed origin (Section 2.1). Twenty plants of both species were used for each age of cutting, which originated from either mainstem or branch material. Each cutting consisted of 2 nodes with the basal leaf pair removed. The numbering of the cuttings started from the basal end of the plant, for example mainstem 1 (MS1) was the most basal cutting on the mainstem and consisted of nodes 1 and 2 with the basal leaf pair removed. Two days before, and straight after striking the cuttings, rovral (0.05%) was sprayed to help reduce fungal attack. Preparation of cuttings was carried out within a fog bench environment. After being dipped in a 1,000 μM IBA solution (50:50 ethanol and water) for 5 seconds (this 'optimum' treatment was developed by Advanced Technologies (Cambridge) Ltd.), the cuttings were placed in a fog bench (base temperature 26-27°C) and harvesting took place 6 weeks later.

In *E. globulus* there was a very sharp decline in the ability of cuttings to root with increasing stock plant age. When the stock plants were 16 weeks old over half the mainstem cuttings formed roots, whereas with the 20 and 25 week old material there was virtually no rooting (Table 3.6). From the limited data it appeared that material originating from the branches did not root well

(Table 3.6). The branch cuttings of *E. grandis* rooted much more readily with increasing stock plant age compared to the equivalent cuttings of *E. globulus*. The rooting percentage of branch origin *E. grandis* cuttings from 20 and 25 week old plants were of very similar rooting ability to each other (Table 3.6). Cuttings from *E. globulus* and *E. grandis* stock plants differed greatly with regard to their rooting percentage with increasing stock plant age, this was particularly apparent in branch-origin cuttings (Table 3.6).

Table 3.6 The rooting percentage of mainstem (MS) and branch (B) cuttings from *E. globulus* and *E. grandis* stock plants of different ages.

***E. globulus.*** Rooting percentage.

Cutting type.

| Age (weeks) | MS1 | MS2 | MS3 | MS4 | B1 | B2 | B3 | B4 |
|---|---|---|---|---|---|---|---|---|
| 16 | 55 | -- | -- | -- | -- | -- | -- | -- |
| 20 | 5 | 10 | -- | -- | 0 | 10 | -- | -- |
| 25 | 0 | 0 | 5 | 10 | 0 | 5 | 0 | 0 |

***E. grandis.***

| Age (weeks) | MS1 | MS2 | MS3 | MS4 | B1 | B2 | B3 | B4 |
|---|---|---|---|---|---|---|---|---|
| 16 | 50 | 10 | -- | -- | -- | -- | -- | -- |
| 20 | 15 | 40 | -- | -- | 60 | 50 | -- | -- |
| 25 | 5 | 15 | 10 | 10 | 65 | 60 | 50 | 30 |

Key:

The numbering system begins at the basal end of the plant, for example MS1 is the most basal pair of nodes on the main stem. Figures given are as a percentage of the cuttings struck that formed roots, there were 20 cuttings per cutting type each from a different plant.

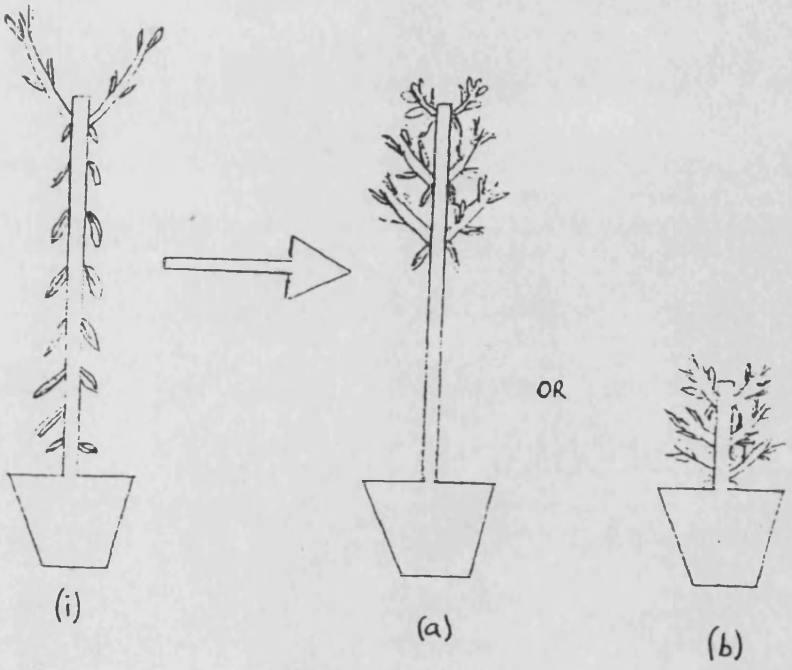
-- cutting not available due to the plant size being too small (the protocol is for semi-hardwood cuttings and is not suitable for soft, fleshy cuttings).

## 3.6 The rooting of cuttings from upper or lower positions of glasshouse-grown *E. globulus* stock plants.

In experiment 3.5 considerable variability was noted between cuttings of different ages, for example the cuttings from the basal end of the younger stock plants were subjected to a lower light intensity than those taken from the more apical regions of the older stock plants. This experiment aimed to investigate further the percentage of cuttings that form roots from different positions on the stock plants and to see if there was a correlation between position and rooting ability. Cuttings were taken from branch material of epicormic origin, in order to obtain a more uniform physical characteristic between cuttings from the upper/lower positions. The cuttings should originate from material of the same absolute age as the epicormic branch material should ensure an even flush of growth; this is in contrast to the previous experiment where branch and mainstem developed as the plants grew.

Six weeks after sowing the seeds, 60 uniform plants were selected and potted on. Eight weeks later all plants were pruned to 1m, all branches were removed except the apical pair which were pruned back to 2 nodes,. After a further 3 weeks the plants were pruned back according to whether shoots were to be encouraged at the base or apex of the plant (Figure 3.8). Two weeks later the plants were repotted and epicormic buds pinched out twice a week to ensure an even flush of growth. The cuttings were taken after a further 12 weeks (31 weeks from sowing). The selection of the 20 stock plants to be used for the cuttings was carried out by picking the plants with the most branches suitable for striking cuttings. Two days prior to striking cuttings the supplementary lighting was turned off. Two days before and immediately after striking the cuttings, rovral (0.05% w/v) was sprayed to help prevent fungal attack. In the

Figure 3.8 The pruning of *in vivo E. globulus* stock plants to encourage shoots at the apex or base of plants.




Key: three weeks after de-branching (i), plants were pruned to encourage shoots either at the apex (a) or, base (b).

fog bench, (base temperature 26-27°C), 4 randomised blocks, each containing the cuttings of ten plants (5 tall, 5 short) were set up. Five replicates of each plant type were used to record the class of branching of the cuttings (primary, secondary, or tertiary). Cuttings, which came from the basal ends of branches, were prepared within the fog bench to minimise water loss. Each cutting consisted of three node pairs, with the basal leaf pair removed. A 50:50 mixture of fine sand and course sand was contained in Plantpak P50 units, each measuring 3 x 4 x 5 cm deep, every other cell was left empty to aid air flow. Seedtrays were used to contain the Plantpak units, and each seedtray was placed 3 cm deep into the fog bench sand to ensure a more even temperature at the base of the cuttings. For each cutting, fresh weight, measured on a balance enclosed in a sealed plastic bag in the fog bench, overall length, and shape of the base, (round, square, or intermediate), was recorded. Cuttings were harvested 6 weeks after striking them.

Of the apical cuttings only 6.5% rooted, whereas 28% of the basal cuttings rooted ($x^2$<0.02). There are numerous factors which could affect the rooting process, for example the cuttings from the apex were larger than those from the basal region. Also all the cuttings from the basal regions had round bases, whereas of the cuttings from the apical regions, 60%, 34%, and 6% were round, intermediate, and square respectively. In following up this experiment the block design could be changed to allow the rooting ability of individual genotypes to be evaluated. In this experiment there appeared a large amount of variation in the rooting ability between different genotypes, however as the block design did not permit analysis on this level it cannot be determined whether the difference rooting ability was due to position in the fog bench or the stock plant genotype.

## 3.7 The effect of position in the stock plant and genotype on the subsequent rooting of glasshouse-grown *E. globulus* cuttings.

This was a follow on experiment from experiment 3.6 designed to permit the genotypes rooting ability to be analysed. The same 20 stock plants used in experiment 3.7 were again used. After allowing the plants to grow and recover there branches were pruned back as before in experiment 3.6. The stock plants were 45 weeks old by the time suitable cuttings could be obtained and it was autumn. The same procedure as used in experiment 4.6 was used. The block design was completely randomised.

Only 3% of the cuttings rooted. There are several reasons why the rooting ability may have been so low. Firstly the fogging unit had not been functioning all that well and many cuttings ended up rotting. Secondly as the stock plants were several months older, it is possible that they had matured too far. Also the time of year was not very good, by the time these cuttings could be excised it was autumn, although there was supplementary lighting conditions were still not that conducive to rapid growth of the stock material. Although the plants appeared healthy and nutrients had been supplied every three weeks, a lack of nutrients could have been a part of the reason for the low rooting frequency. Due to a lack of time and lack of space in the fog bench this experiment could not be repeated.

## Rooting discussion.

From the present rooting studies with *E. globulus* it is clear that the rooting ability can decline very rapidly with age, this is consistent with other work (Hartney 1982 and Duranz 1988). In experiments 3.1a and 3.1b, which used magentas with water as the rooting medium, none of the four week old cuttings rooted (Figure 3.1, 3.3). Seedlings germinated *in vitro* and placed in a tissue culture medium (Experiment 3.2a,b and 3.3) had a less pronounced decline in rooting ability compared to the ones rooted in water (Experiments 3.1a,b).

The difference between experiments 3.2a and 3.2b was the type of container used. Boiling tubes were used in experiment 3.2b in order to permit 24 replicates compared to six with the magenta system (Experiment 3.2a). In boiling tubes the rooting percentage declined at a much slower rate than in magentas. In boiling tubes the rooting percentage of 21 and 28 day old explants was 95.3% and 97.17% respectively (Figure 3.5b), whereas in magentas it was 41.6% and 37.5% respectively for these two ages (Table 3.3). Conditions in the two types of container could possibly explain the difference in rooting ability. A similar difference in the rooting percentage between the two types of container was also evident in experiments 3.2b and 3.3.

Application of IBA also enhanced the rooting ability of cuttings (Experiment 3.4), but this only has a limited use as with more mature explants the rooting ability can still decline significantly with age (Experiment 3.3, 3.5).

From the present work with *E. globulus* it is apparent that the phase change is very rapid, the rooting percentage can decline significantly in a

matter of only a few weeks, and this makes *E. globulus* a good model for this type of work. If etiolation does effect the subsequent rooting of cuttings, it is not that likely to be for histological reason as the decline in rooting ability can be so rapid that there is little time for large changes in histology. There does not appear to be any reference to the effect of shoot etiolation on the subsequent rooting of *E. globulus*. It could prove useful to try this approach with *E. globulus*, in particular with old material that does not root, or where the rooting percentage is very low with auxin application, as etiolation in conjunction with auxin application has resulted significantly higher rooting rates than in the light-grown controls with M.9. apple rootstock (Harrison-Murray, 1982). Another potentially useful approach would to etiolate epicormic shoots from the base of adult trees, as epicormic shoot have shown the ability to form roots in many eucalypts (Hartney, 1980).

The majority of the rooting data obtained in this thesis involved stem cuttings. The advantages of stem cuttings are that a large number of cuttings can be obtained from a single tree, the problem of graft incompatibility is avoided and that it is far less time consuming than grafting or layering and therefore cheaper. Cuttings taken from young eucalypt seedlings usually root well (Cresswell *et al.*, 1982; Mc Comb *et al.*, 1986). However there are exceptions, for example young seedling *E. globulus* cuttings gave less than 30% rooting (Hartney, 1982). The decline in the rooting ability of *E. globulus* cuttings with increasing stock plant age was very apparent from the rooting data in this thesis. As the stock plants aged the rooting ability of cuttings declined sharply, especially without applied auxin treatments. The application of IBA improved the rooting ability of cuttings, but it only delayed the age-

related decline in rooting ability i.e. when IBA was applied to cuttings of increasing age, their rooting ability still declined, but over a longer period of time compared to those rooting without applied auxin treatments.

Maturation is a highly complex developmental process which includes changes in morphological and physiological processes leading to the reproductive (mature) state (Hackett 1987). Associated with the transition from the juvenile to mature state are progressive changes in morphological and developmental characteristics including the leaf cuticle, bark, leaf shape, leaf thickness, phyllotaxis, vigour of shoot growth and stem pigmentation. Changes in such characteristics during development vary from species to species and most changes occur gradually during the period preceding maturity. With the present work using *E. globulus,* certain characteristics associated with the onset of maturation, for example leaf shape changing from round to strap-like and stem shape changing from round to square, occurred between 20 and 25 weeks after germination. The rooting ability of glasshouse-grown *E. globulus* and *E. grandis* cuttings varied considerably with age. The decline in the rooting percentage was more pronounced in *E. globulus* than *E. grandis* (Table 3.2). With 16 week-old *E. globulus* stock plants over half the mainstem cuttings formed roots, whereas with 20 and 25 week old material there was virtually no rooting (Table 3.2). These results are in agreement with the general view that *E. globulus* is a more difficult to root species than *E. grandis* (Hartney, 1981; Mc. Comb and Bennett 1986). Conflicting results on the rooting ability of different species of *Eucalyptus* do however exist, for example Gupta and Mascarenhas (1987) working with mature elite trees of *Eucalyptus* species, reported that *Eucalyptus camaldulensis* explants did not root as readily as *E.*

*globulus* explants; most work describes *E. camaldulensis* as an easy to root *Eucalyptus* species (Giordano, 1961; Hartney and Barker, 1980; Heth *et al.*, 1985). The present work using *E. globulus and E. grandis* helps to reinforce the description of maturation as a complex developmental pathway with associated changes in morphological and developmental characteristics. Due to the large amount of variation noted between cuttings of different ages in experiment 3.5, plants were pruned to encourage shoots either at the apex or base in order to obtain a more uniform cutting type. Basal cuttings had a significantly higher ($p<0.02$) rooting ability than the apical cuttings. The position of cuttings on the mother plant has been known to have an effect on the rooting ability for sometime. Cuttings from lower branches of conifers, especially in positions near the trunk, are more juvenile than branches in other parts of the tree (Bonga 1987). Similar positional effects have also been noted in *Eucalyptus* (Cresswell *et al.*, 1982).

Despite much work there are still no clear answers to basic questions such as where in the cell or tissue, and how, does maturation occur? Part of the problem is the complexity of the developmental process leading to the mature state. Structural differences have, in some species, been cited as a factor affecting rooting. However, such evidence is certainly very varied. White and Lovell (1984b) concluded that *Agathis australis* cuttings from old material contain abundant resin canals, schlerenchyma and branch traces, and that these may have reduced the amount of parenchyma tissue to such a low concentration that there were no longer potential sites for root initiation and root formation was prevented. Davies *et al.*, (1982) suggested that the anatomical dissimilarities between juvenile and mature stems of *Ficus pumila*

did not account for differences in ARF. Perivascular sclereids (macrosclereids) were thicker in mature stems, but primordia penetrated these with relative ease. The work on *E. globulus* in this thesis showed how rapidly the loss in rooting ability took place. Of the seedling material that did not root, no root primordia were seen to be physically prevented from penetrating across the cortex when hand sectioned. The glasshouse-grown *E. globulus* and *E. grandis* stock material had much more thickening than the very young seedling material used. With the 1,000 μMol IBA dip, 55% of the 16-week old *E. globulus* cuttings rooted (Table 3.6), it appeared that roots were able to emerge with ease as no semi-developed root primordia were seen when the base of the cuttings were hand sectioned. Another indication that root primordia were not physically prevented from extending across the cortex was with the young seedling material, rooted without auxin application. Here the decline in rooting with increasing stock plant age was rapid, no discernible physical barriers to root growth were apparent and when the non-rooting material was sectioned no partially developed root primordia were detected. In addition it was possible to recover high rooting percentages by application of auxin to the rooting medium.

Numerous workers (Bonga 1982a; Hackett 1985,1987; Greenwood 1987; Pierik 1990) have discussed whether the stability of the mature state is determined at the concentration of the individual cell, the entire apical meristem, or is due to correlative effects involving the whole plant. These three possible explanations have been termed cellular, structural and correlative respectively (Hackett 1985, 1987; Pierik 1990). Earlier workers, for example Borchert (1976), proposed that increasing structural complexity and size of

growing trees resulted in maturation. However, this has been questioned (Pierik 1990). The evidence for all three possibilities is equivocal, arguments for an important role for meristems is fairly strong, for example when adult meristems are isolated and/or used in grafting experiments they are not easily altered (Greenwood 1984; Bonga 1987). Using *Hedra,* Greenwood (1987) described the stable behaviour of even extremely small grafted mature scions, which nevertheless will exhibit juvenile characteristics after serial grafting or tissue culture. In this case maturation could result from an increase in the proportion of mature cells in the apical meristem. Conversely the previously mentioned methods of rejuvenation could promote relatively more rapid division of vestigial juvenile cells, which gradually increase relative to the mature proportion. Thus simultaneously a cellular basis (where some cells are irreversibly mature) plus explants being able to respond to external stimuli due to remaining juvenile cells dividing more rapidly could exist. *E. globulus* is an ideal candidate for the study of maturation in woody species for several reasons including the rapid reduction in ability to root with increasing stock plant age, the distinct dimorphic characteristics which include leaf and stem shape as the plants age, and the large economic importance of the species which is reflected in the amount of interest in clonal eucalypt forestry programmes.

Strategies to improve the rooting ability of material include hedging, which has been utilised successfully with *E. grandis* and various hybrids in Tunisia, the Congo and Brazil (Mc Comb and Bennett 1986). Shoots which sprout from the stump may also exhibit juvenile characteristics, including the ability to form roots (Mc Comb and Wroth 1986). To develop the desired clonal lines, trees with the required superior characteristics are felled and

selection is made of those individuals that coppice well. Rooting percentages of 80% and above have been reported for *E. grandis* (Campinhos and Ikemori 1977; Chaperon and Quillet 1977). Major problems with the technique of felling trees and using the shoots which sprout from the stump are that the rooting ability of the sprouts may be very variable between different trees, the tree has to be destroyed in order to obtain the shoots, and the amount of shoots that emerge can be variable. Mazalewski and Hackett (1979) used cytokinins to induce buds to break in the lignotuber as well as the upper trunk region of *Eucalyptus ficifolia.* Stem cuttings from the cytokinin-induced shoots exhibited a greater propensity to root when taken from the area of the lignotuber than when taken from higher on the trunk. Furthermore, cuttings from the basal parts of shoots, originating from the lignotuber, rooted better than cuttings taken from the apical portions of these shoots. Several successive subcultures (Gupta *et al.*, 1981) and successive grafting (Siniscaleo and Pavolettoni 1988) have also been used to improve rooting ability; both of these techniques have the problem of being labour intensive. Tissue culture has been used by many workers to try and obtain clonal lines of woody species. The main problems in the micropropagation of eucalypts (Cresswell *et al.,* 1982; De Fossard *et al.,* 1978) are obtaining sterile material from field grown trees and the rooting of shoots from mature trees. Procedures to help overcome the problem of contamination include spraying the shoots on the mother plant with insecticides, etiolating the tissue in order to obtain rapid growth which is relatively clean compared to slower-growing material and the use of small explants such as single nodes (Bonga 1987). The etiolation and use of small explants has been also shown to improve the rooting ability in certain cases

(Howard *et al.,* 1988). The use of small explants has also been cited as a method of reducing correlative controls that exist between different parts of the tree (Bonga 1987).

The above mentioned strategies to improve the rooting ability have only had a limited amount of success. Frequently it is only a few genotypes that respond well to the various rooting treatments. One approach is to select the most juvenile material from the trees of interest and combine some of the strategies mentioned above in order to try and improve the rooting percentage, for example inducing etiolated shoots by dark treatment in the most juvenile region, serial sub-culture and application of auxin. Another approach, which in the long term may be more useful is to investigate the fundamental aspects of ARF and to try and better understand the mechanisms involved. Future directions in the study of ARF have been covered by many workers (Davis and Haissig 1994).

# Chapter 4. Analysis of IAA.

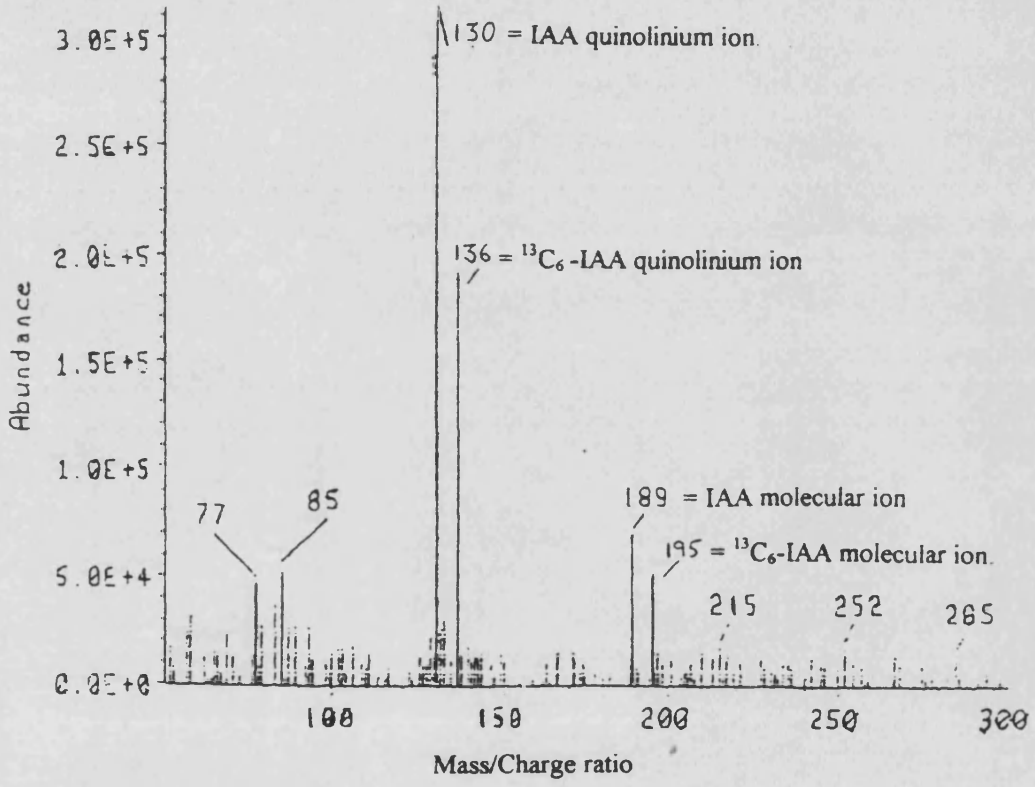
Suitable rooting systems for use in the analysis of endogenous IAA were developed in the work described in chapter 3. For IAA analysis, work will initially focus on developing suitable extraction and purification protocols. Analysis will be carried out using gas chromatography-mass spectrometry (GC-MS), initially employing electron impact-GC-MS which is a well documented technique for the analysis of indoles. The primary focus of the work will be to quantify endogenous IAA concentrations during the main events of ARF. In order to identify the actual times at which to assay, it will be necessary to get information, using histological studies, on the timing of anatomical events, with particular emphasis on the primary events. Time permitting, studies will also be made on material of differing rooting ability to see whether or not there is a relationship between rooting ability and endogenous IAA concentrations in cuttings.

## 4.1 Purification of *Eucalyptus globulus* tissue for the analysis of endogenous IAA.

The aim of this work was to develop a purification procedure for the analysis of endogenous IAA in *E. globulus* shoot tissue. Various plant samples were harvested (Table 4.1) and immediately immersed in liquid nitrogen, freeze dried until no further decrease in dry weight occurred and stored at ⁻70°C. Samples were ground in a mill (Materials and methods 2.4.1) $^{13}C_6$-IAA added, and 30,000 dpm of 2-$^{14}$C-IAA (specific activity 2.04 GBq mmol/l), was added to each sample (Table 4.1). The 2-$^{14}$C-IAA was used as a radiotracer in the early experiments to aid detection of where the IAA was going. The high specific activity results in extremely small amounts of authentic IAA being added to the samples. For rooting data, when we have a reliable system developed, addition of 2-$^{14}$C-IAA will not be necessary. Purification followed the scheme described in the materials and methods 2.4.2 and included solvent partitioning, ion exchange (Sephadex QAE-25), $C_{18}$ Sep-Pak and reverse phase HPLC. The fraction with the same retention time as authentic IAA standard was derivatised using diazomethane prior to GC-MS, which was carried out on a bench-top machine run under electron-impact positive-ion mode.

Monitoring the amount of 2$^{14}$C-IAA indicated that losses occurred during solvent partitioning (40-50%) and ion-exchange chromatography (20-30%). When the GC-MS was running in full-scan mode, the characteristic quinolinium (130 m/z) and molecular (189 m/z) ions from IAA plus the corresponding 136 and 195 (m/z) ions from the $^{13}C_6$-IAA internal standard were present (Figure 4.1). In addition other characteristic ions from IAA were present, m/z 77 and 103 (phenyl and steryl ions respectively ) and the elution time was identical to authentic IAA. The calibration (Figure 2.2 a,b) showed that the $^{13}C_6$-IAA did not contain any authentic IAA. A purified seedling hypocotyl extract (0.5g dwt.), separated by the GC-MS operating in selected-ion monitoring (SIM) mode (Figure 4.2) and the mass spectrum data (Figure 4.1) indicated that the samples were sufficiently pure to give reliable results. There were however other ions present even with SIM (Figure 4.2). Sizeable contaminant

Figure 4.1 EI-GC-MS mass spectrum of the putative methyl ester of IAA from 20 day old *Eucalyptus globulus* seedling material (0.5g dry mass). Internal standards of 100 ng $^{13}C_6$-IAA and 30,000 dpm 2-$^{14}$C-IAA were added.



195 ions were detected, but these were sufficiently separated from IAA to avoid interference (Figure 4.2). No conclusions on quantification between different samples were drawn from the data (Table 4.1) as there was no replication, the purpose of the work was to obtain clean samples with a suitable level of internal standard for IAA analysis. This experiment indicated that 150ng-250ng of $^{13}C_6$-IAA and 30,000 dpm of 2-$^{14}$C-IAA was a suitable level of internal standard for this type and quantity of material.

Table 4.1 Amount and type of material, old (3 month primary branch origin), seedling (20 day old), used for extraction and the quantity of $^{13}C_6$-IAA added as internal standard.

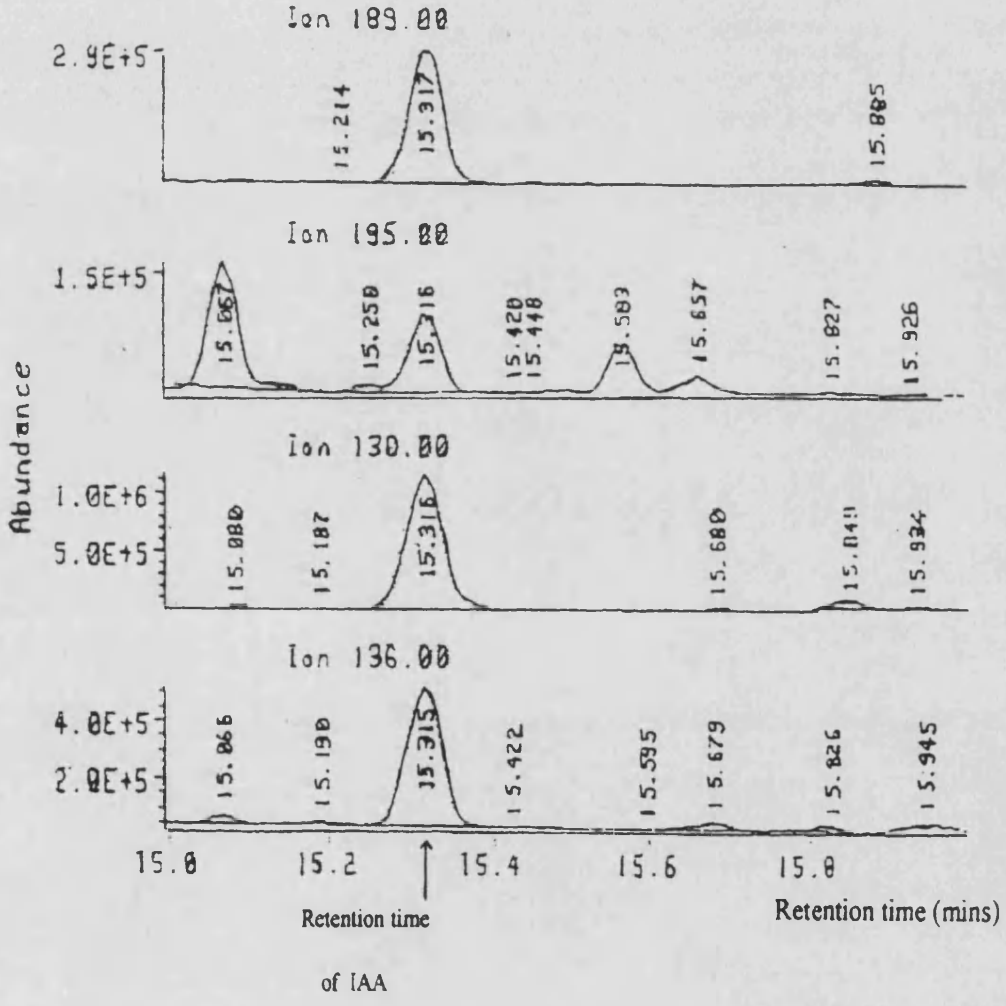
| Tissue type And dry mass (g) | Mass $^{13}C_6$-internal standard (ng) | Endogenous IAA (ng/g dry mass) m/z 130/136 | m/z 189/195 |
|---|---|---|---|
| 1. Old stem (3) | 250 | 209 | 283 |
| 2. Seedling (0.5) | 100 | 664 | 780 |
| 3. Seedling (0.5) | 100 | 420 | * |
| 4.Seedling hypocotyl (0.05) | 100 | * | * |
| 5. Seedling hypocotyl (0.5) | 250 | 835 | 875 |

Key:

* Denotes where the ion ratio is too low.

With the inclusion of solvent partitioning, ion-exchange, $C_{18}$ Sep-Pak and HPLC, purification was a lengthy process and losses were high. In order to purify a large number of samples, a simpler procedure would be desirable. The intensities obtained from the hypocotyl extract (Figure 4.2) gave an indication that much smaller quantities of plant tissue could be analysed.

Figure 4.2 Typical EI-GC-MS-SIM trace of the putative methyl ester of IAA from 20 day old *Eucalyptus globulus* seedling material (0.5g dry mass). Internal standards of 100 ng $^{13}C_6$-IAA and 30,000 dpm 2-$^{14}$C-IAA were added.



## 4.2 The importance of solvent partitioning in the purification of *Eucalyptus globulus* seedling cuttings for IAA analysis.

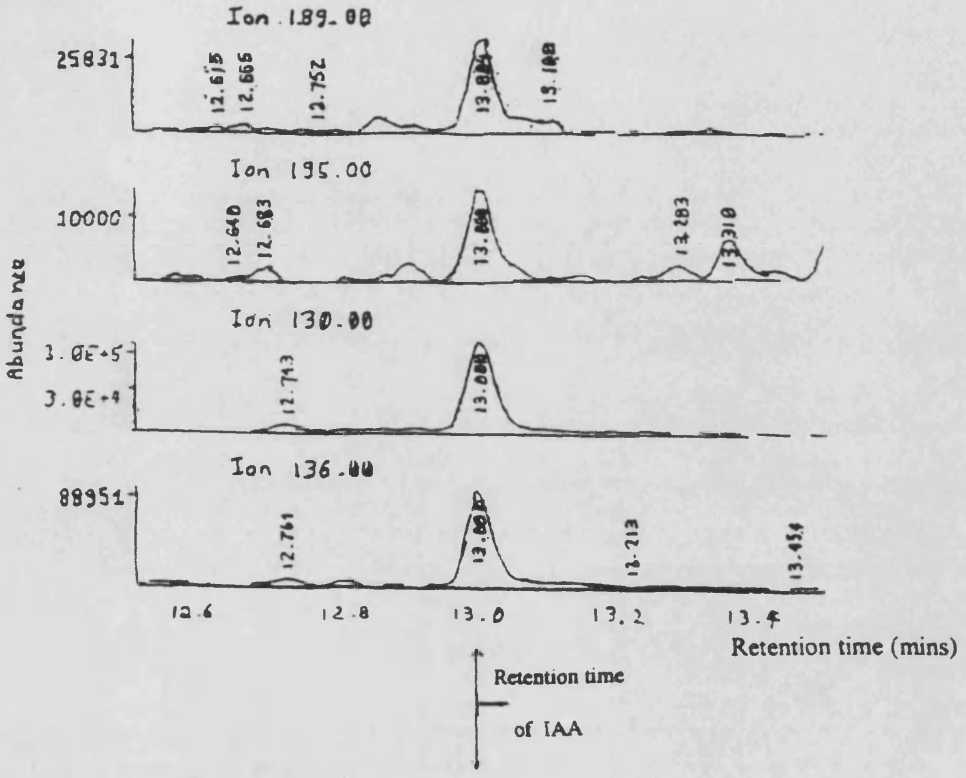
The aim of this experiment was to find a simpler purification system than that used in experiment 4.1, not only to reduce the amount of time necessary for sample purification but also to allow smaller amounts of tissue to be used. As solvent partitioning resulted in high losses (40-50%) of $2^{14}$C-IAA (Experiment 4.1) it was decided to determine the necessity of solvent partitioning in the purification of seedling material for IAA analysis.

Twelve-day old seedlings were severed just above the soil surface, immersed immediately in liquid nitrogen and then freeze-dried. Four replicate samples, each weighing approximately 0.42 g dry mass, were extracted in 80% MeOH with 100ng of $^{13}C_6$-IAA and 30,000 dpm of 2-$^{14}$C-IAA added to each sample. Following extraction, the purification procedure included sephadex, Sep-Pak and reverse phase HPLC (Materials and methods 2.4.2)

The purification procedure, which omitted solvent partitioning, yielded quantifiable results with three of the four replicate samples (Figure 4.3i). Here, the characteristic ions from the methyl ester of IAA can clearly be seen and although there are ions from other sources present, they are much less abundant and separated by elution time. The omission of solvent partitioning can however lead to problems, as seen in one of the four replicate samples (Figure 4.3ii). The number of contaminant ions from the methylation of other compounds is large, the time separation is poor and in addition the peak shapes are poor (Figure 4.3ii). Although there is considerable variability in the y axis between figures 4.3i and ii, it is the ratio of 130/136 and 189/195 ions which is used to calculate the mass of IAA, and therefore losses are automatically accounted for. There was reasonable agreement for the 3 replicates, but the more reliable estimate should come from the 130/136 ion ratio, as the peak intensity is much greater. The results indicate concentrations of IAA ranging from 489 to 530 ng/g dry mass in the 3 replicate samples (Table 4.2). The difference in the

Figure 4.3 Typical EI-GC-MS-SIM traces of non-solvent partitioned *E. globulus* seedling material (0.42g dry mass). Internal standards of 100ng $^{13}C_6$-IAA and 30,000 dpm of 2-$^{14}$C-IAA were added.

i) non-solvent partitioned




ii) non-solvent partitioned

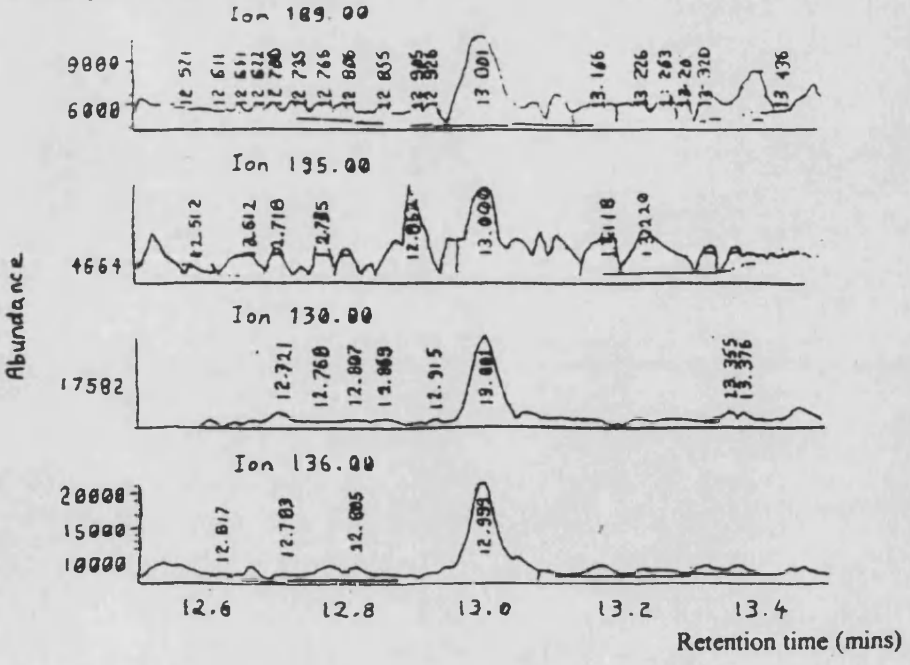
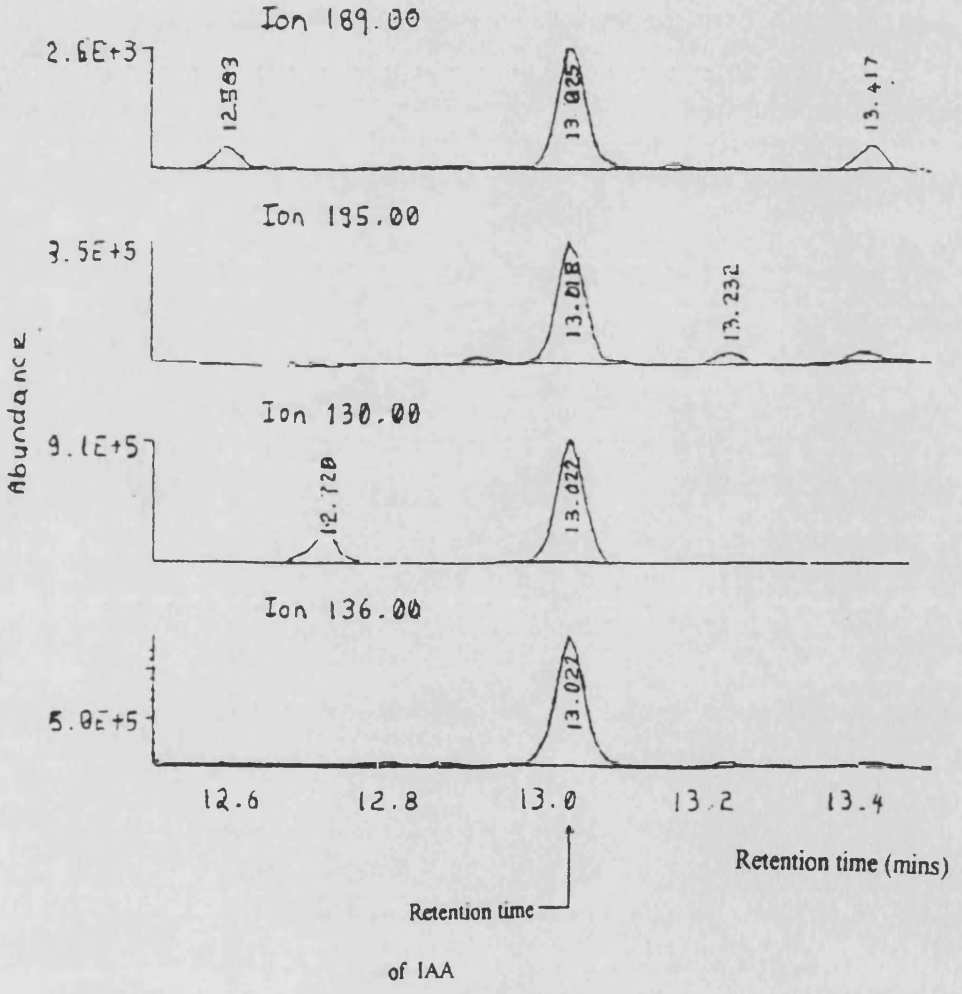


Figure 4.4 Typical EI-GC-MS-SIM trace of solvent partitioned *E. globulus* seedling material (0.42g dry mass). Internal standards of 100ng $^{13}C_6$-IAA and 30,000 dpm 2-$^{14}$C-IAA were added.



retention time between results for example, figures 4.2 and 4.4, is not unexpected as factors such as the column length (which varies when parts are cut off the leading end for cleaning purposes) and the carrier gas flow effect retention time.

Table 4.2 Endogenous IAA concentrations (ng/g dry mass) from *E. globulus* seedling cuttings using EI-GC-MS-SIM, omitting solvent partitioning in the purification procedure.

| | Mass IAA (ng/g dry mass) | |
|---|---|---|
| Replicate | 130/136 | 189/195 |
| 1. | 530 | 500 |
| 2. | 489 | 415 |
| 3. | 508 | 610 |
| Mean | 509 +/- 20 | 508 +/- 98 |

Key:

+/- standard deviation.

From this data it seems best to include the solvent partitioning stage when using this type and amount of tissue. Although quantifiable data can be obtained using the shorter purification procedure, especially using the quinolinium ions (130 and 136), it nevertheless can yield data which is not always that clean. If the elution time of the non-IAA ions (Figure 4.3ii) varied even slightly it could easily interfere with the analysis.

## 4.3 IAA concentrations in *E. globulus* seedling cuttings at three time periods during the rooting process.

The purification procedure described in the previous experiment (4.2) was used again, with solvent partitioning still omitted due to the smaller sample size used here. Endogenous IAA was analysed in 0.27g dry mass of young seedlings by GC-MS at three time periods during the rooting process. Twelve day old seedlings were severed just above the soil surface, struck in vermiculite and kept in a Saxcil cabinet running at 25°C with continuous lighting. Three replicate batches of twenty five seedlings (0.27g dry mass) were used, harvesting took place at three times 0, 24 and 48 hours after cutting excision. After grinding in 80% MeOH, 50ng $^{13}C_6$-IAA (reduced from the 100ng level in experiment 4.2 due to the smaller amount of plant material being used here) and 30,000 dpm of 2-$^{14}$C-IAA was added.

Losses estimated using 2-$^{14}$C-IAA during purification were high, approximately 70%, (50% during Sephadex ion-exchange and 20% during HPLC). This resulted in small peaks; the original level of internal standard was low because it had been assumed that with Sephadex, $C_{18}$ Sep-Pak and HPLC, losses would be significantly lower than when the solvent partitioning was incorporated (Experiment 4.1). In most samples the intensity of the 189/195 m/z ions was insufficient to give an accurate estimation of IAA concentrations (Table 4.3). High levels of background noise were present in many samples, this combined with rather low levels of internal standard resulted in a low signal to noise ratio. With material such as seedling hypocotyl, rather than seedling material which has the pigmented shoot system, this purification method might be more suitable. With the seedling material used in this experiment, solvent partitioning might be necessary.

Table 4.3 Endogenous IAA concentrations using EI-GC-MS-SIM from 12 day old *in vivo E. globulus* seedling cuttings at three times after cutting excision.

| | Mean mass IAA (ng/g dry mass.) | |
|---|---|---|
| Time after cutting excision | 130/136 | 189/195 |
| 0 hours | 602.5 (+/- 37.5) | 600* |
| 24 hours | 541 (+/- 431.3) | ---- |
| 48 hours | 652 (+/- 73.5) | 815* |

Key:

number = 3 replicates.

+/- number denotes the standard deviation.

* n = 2

## 4.4 (a) Analysis of endogenous IAA in *E. globulus* seedling hypocotyl material and the importance of ion-exchange Sephadex and HPLC.

Following the results of experiment 4.3 it was decided to use hypocotyl tissue instead of hypocotyl cuttings which included the shoot system, as adventitious roots originate in the hypocotyl. As the material used in this experiment appeared much less pigmented, solvent partitioning was again omitted and the importance of Sephadex QAE-25 and HPLC was tested.

Ten 1cm hypocotyls in each of 5 replicates were harvested and immediately immersed in liquid nitrogen, then freeze-dried. During extraction, 25ng of $^{13}C_6$-IAA and 30,000 dpm of 2-$^{14}$C-IAA internal standard was added to each sample. Purification ( Materials and methods 2.4.2) methods were:

1,2. Sephadex, Sep-Pak, with/without HPLC

Sephadex

Sep-Pak $C_{18}$

+/- HPLC (GC was used before and after, on the same extract).

3. Sep-Pak and HPLC

Sep-Pak $C_{18}$

HPLC

Five replicates were used for each purification protocol.

Due to the presence of a contaminating 195 ion in samples without HPLC purification (Figure 4.5), all samples had to go through this step before reliable GC-MS data could be obtained. In the absence of HPLC there was an additional problem of poor peak shape . Incorporation of the HPLC purification step eliminated the contaminating 195 m/z ion and resulted in SIM traces which were clean with a good peak shape (Figure 4.6). With the hypocotyl material purified here, the use of Sephadex was not necessary as the SIM traces were clean with a good peak shape. Not only did this save time, it also resulted in a large decrease in the loss of 2$^{14}$C-IAA. The losses during the Sephadex purification step were typically around 35-40%, consequently, omitting this step would allow considerably smaller quantities of tissue to be used. The 'rooting zone' in 12-day old seedling material consisted of the basal 2.5mm of hypocotyl (Experiment 3.2a). The ten 1cm hypocotyls used in this

Figure 4.5 Typical EI-GC-MS-SIM trace of *E. globulus* seedling hypocotyl origin material without HPLC purification. Sample from seedling hypocotyl material with 25 ng $^{13}C_6$-IAA added as internal standard.

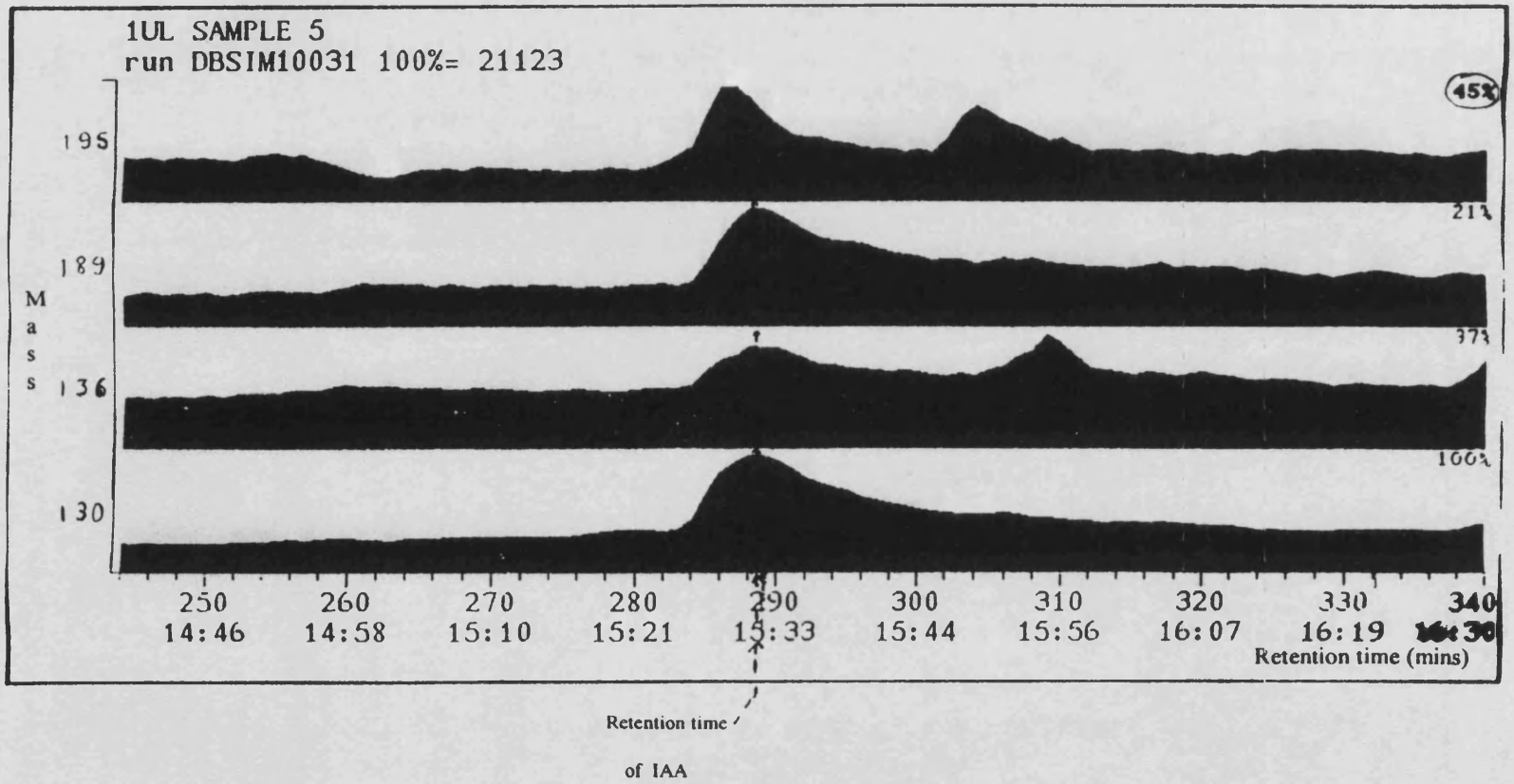
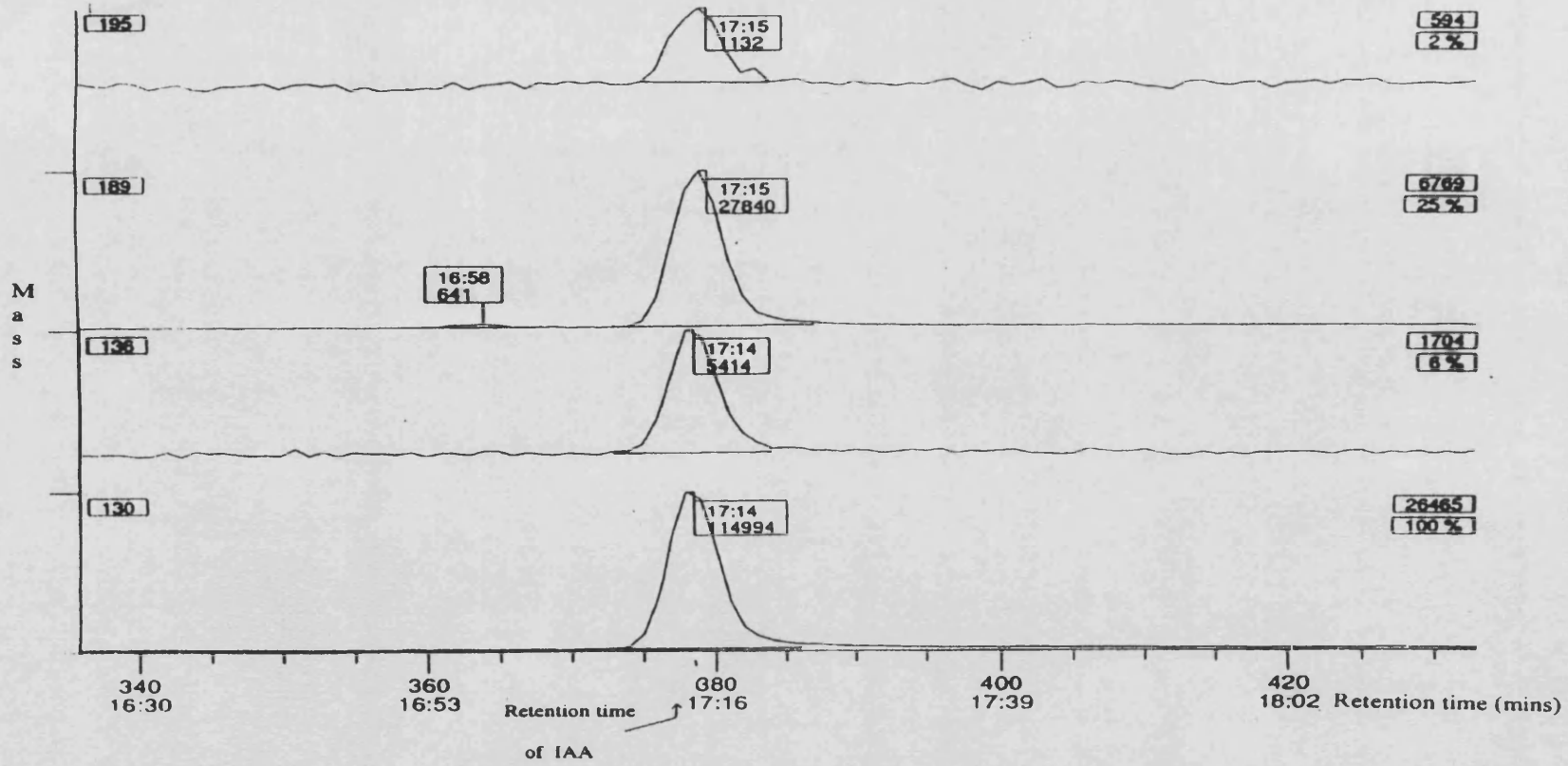


Figure 4.6 Typical EI-GC-MS-SIM trace of *E. globulus* seedling hypocotyl origin material inorporating HPLC purification and omitting sephadex. Samples from seedling hypocotyl material with 25 ng $^{13}C_6$-IAA added as internal standard.



experiment therefore represent the equivalent mass of 40 rooting zones. The time saved in purification, plus the lower losses resulting in less material at extraction being necessary, could allow measurement of endogenous IAA concentrations in the rooting zone over many time periods to be practical.

## 4.4 (b) Quantitative analysis of endogenous IAA concentrations in 12 day and 30 day old *E. globulus* hypocotyl material.

Glasshouse grown seedling hypocotyls were harvested into liquid nitrogen and then freeze-dried. The 12 day old material readily forms roots whereas the 30 day old material does not when rooted in water (Experiment 3.1a,b). Forty six young hypocotyls (0.03g dry mass) and 27 old hypocotyls (0.12g dry mass) were used for each of the two ages, and 3 replicates were analysed. 10ng of $^{13}C_6$-IAA was used as an internal standard, purification included $C_{18}$ Sep-Pak and reverse phase HPLC following the results of the previous experiment.

Variability in the data (Table 4.4) was very small, this is true not only between replicates but also within replicates when the ratios of the quinolinium (130/136) and molecular ions (189/195) are compared. Whilst this data is for relatively large amounts of plant material, whole hypocotyls rather than 2.5mm hypocotyl rooting zones, it nevertheless gives an indication of the endogenous IAA concentrations in the plant material. The 12 day old material, which does form roots, had significantly ($P<0.01$) more IAA per gram dry mass of hypocotyl than the 30 day old non-rooting material. The results are in good agreement with those from earlier experiments with the exception of experiment 3.1, which had twice the amount of IAA, but in that experiment there was only one replicate.

Table 4.4 IAA concentrations (ng IAA /gram dry mass of hypocotyl) in 12 and 30 day old *E. globulus* seedling hypocotyl material using EI GC-MS-SIM.

| | Mean mass IAA | (ng/g dry mass) |
|---|---|---|
| Sample | 130/136 | 189/195 |
| 12 day old | 400.0 (+/- 57.7) | 500.0 * |
| 30 day old | 248.3 (+/- 18.7) | 244.2 (+/- 23.2) |

Key:

(+/- number) denotes the standard deviation).

* denotes missing values due to low 189 m/z ion peak.

## 4.5 Qualitative analysis of endogenous IAA in the rooting zone of 14 day old *E. globulus* seedling cuttings.

The rooting zone in young seedling hypocotyl cuttings consisted of the basal 2.5mm of the hypocotyl (Experiment 3.2a). The simple, rapid purification system developed for small amounts of hypocotyl, as described previously in section 3.4a was used here for analysis of IAA in the rooting zone.

One cm of hypocotyl was used to obtain 2.5mm sections, each 2.5mm section being the equivalent mass of a 'rooting zone'. Three replicate samples containing 10, 50 and 100 'rooting zones' were harvested into liquid nitrogen and freeze dried. Each 'rooting zone' weighed approximately 0.2mg dry mass. The purification procedure incorporated simply $C_{18}$ Sep-Pak with or without HPLC ( Materials and methods 2.4.2iii,iv).

$^{13}C_6$-IAA internal standard was not used here so that background 136 and 195 ions could be checked. Radiotracer was not used as the purification procedure was simple, and also to give an opportunity to confirm unequivocally the presence of endogenous IAA. With all samples HPLC was necessary for adequate purification to enable GC-MS-SIM monitoring. Without the HPLC step, contaminating 195 ion was detected which co-eluted with the 130 and 189 ions from the IAA (Figure 4.7). Also omitting the HPLC step resulted in poor peak shape, as seen in the tailing. After HPLC the SIM traces were very much different. The contaminating 195 ion was not present, the other background signals were minimal and the peak shape was good (Figure 4.8). The samples were checked for authenticity by firstly checking the elution time against authentic IAA and secondly running the GC-MS in high resolution mode. The samples originating from 10 rooting zones contained insufficient IAA for quantification (1µl from 10µl was injected). Both 100 and 50 'rooting zone' samples contained adequate concentrations of IAA for quantification. A potential problem is insufficient IAA during the later stages of ARF, during which times workers have found low auxin concentrations (Blakesley *et al.*, 1991a). If very low concentrations are the case it is possible to double the injection size and/ or use more rooting zones

Figure 4.7 Typical EI-GC-MS-SIM trace of *E. globulus* seedling hypocotyl origin material without HPLC purification (samples had no $^{13}C_6$-IAA internal standard).

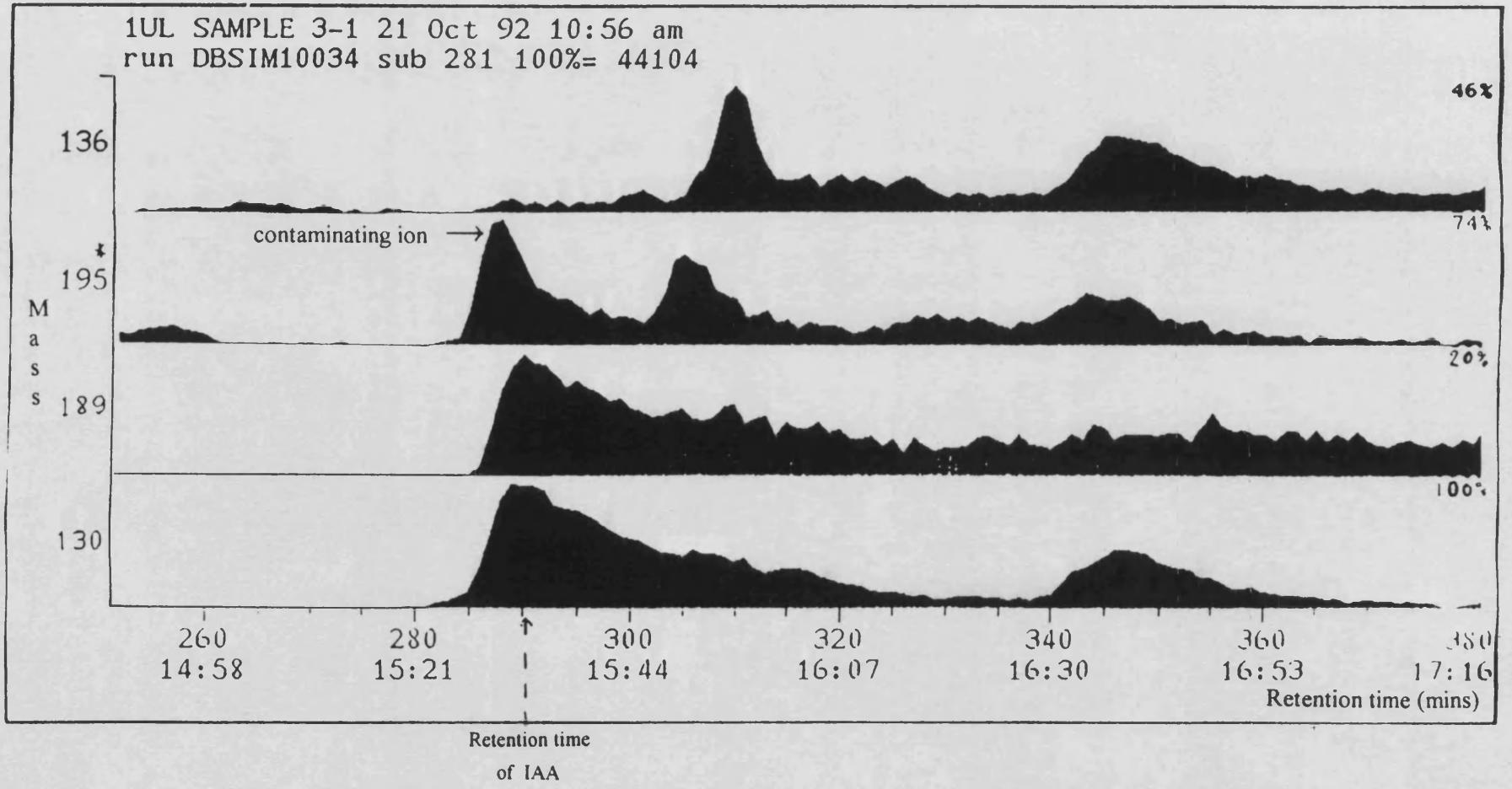
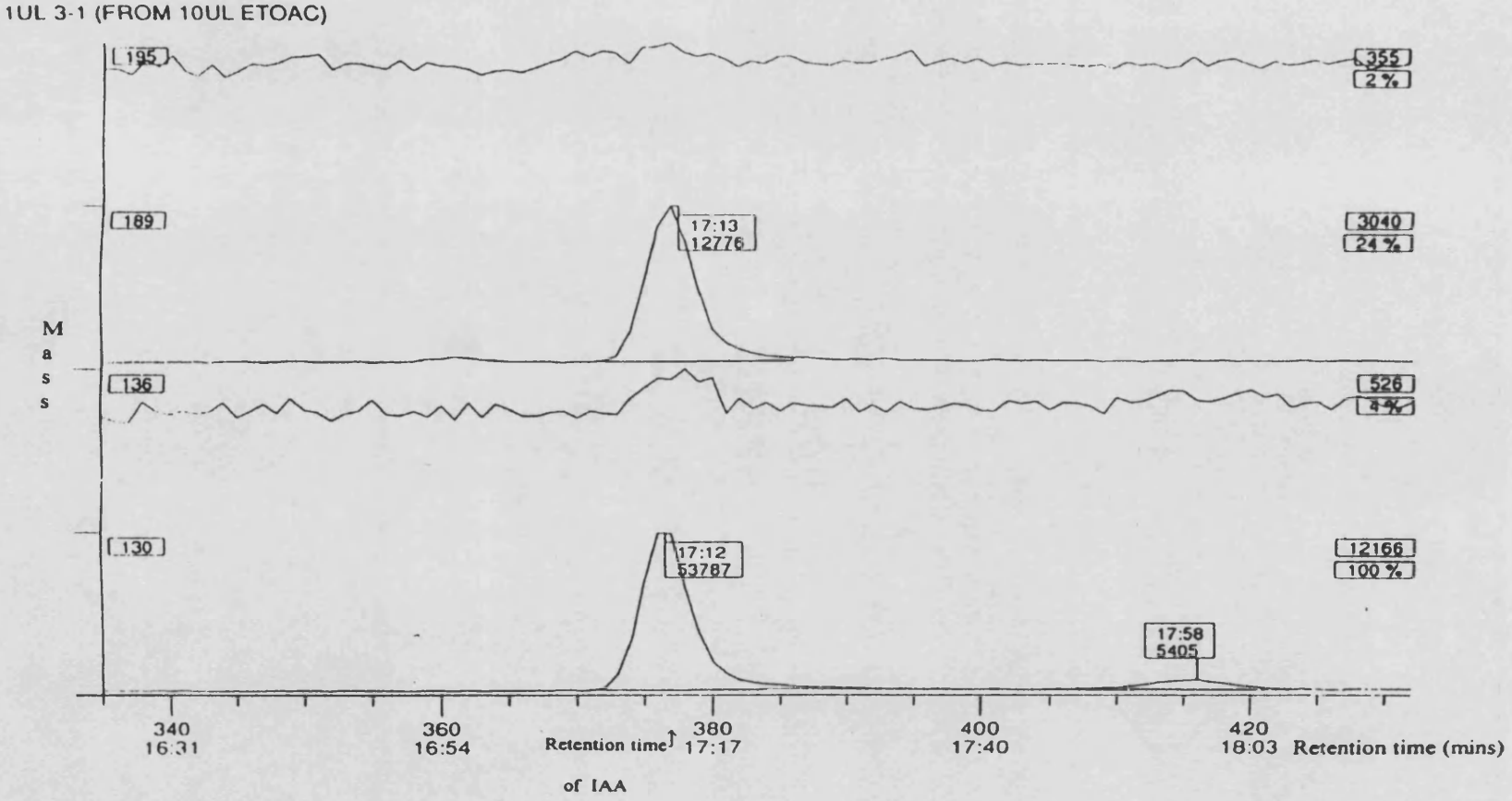


Figure 4.8 EI-GC-MS-SIM trace showing removal of contaminating 195 m/z ion following purification by HPLC.



for this period. The next step is to attempt to quantify IAA concentrations in the rooting zone of 12 day old seedling cuttings using $C_{18}$-Sep-Pak and reverse phase HPLC as the purification protocol.

## 4.6 Endogenous IAA concentrations during the rooting of *Eucalyptus globulus* seedling cuttings.

The aim of this experiment was to identify the histological sequence of events during ARF and to monitor endogenous IAA concentrations associated with the various stages in the rooting process. Cuttings used in this experiment were not exposed to exogenous auxin.

Anatomy.

Fourteen-day old seedlings were transferred from the glasshouse to Saxcil cabinets where the cuttings were kept for 3 days prior to cutting excision to allow them to adjust to the conditions. Cuttings, prepared by severing the hypocotyl 1cm below the cotyledons with a sharp razor blade, were struck in vermiculite. The cuttings were kept at 25°C, with continuous lighting. Cuttings were subsequently harvested 0,10,20,36,48,60,72,84 and 96 hours after cutting excision. The 10 replicate hypocotyls were cut with a sharp razor blade and immersed immediately into FAA, at each harvest. After dehydration and embedding, sections were stained in either safranin-fast green or toluidine blue.

Hormone Analysis.

The basal 2.5mm and upper 2.5mm of the severed hypocotyl were harvested for hormone analysis; these represented the rooting zone and a non-rooting zone respectively. Three replicate batches of 30 cuttings were harvested. Material was harvested with a sharp razor blade and immersed immediately into liquid nitrogen, after which it was freeze-dried. The internal standard, 2ng of $^{13}C_6$-IAA, was added at the start of the extraction. Following purification, derivatisation was carried out using diazomethane. EI GC-MS in full scan mode was used for identification and in SIM mode for quantification.

**Anatomy.**

In the rooting zone the first visible sign of ARF occurred after 35 hours when the nuclei of a few cells in the pericycle associated with the four vascular bundles were noticeably swollen and prominent (Plate 4.1b), in contrast to 0 and 20 hours (Plate 4.1a). By 45 hours the number of cells, in close association with the vascular bundles, that had swollen prominent nuclei had increased, and early cell division was underway (Plate 4.1c). At this stage no organised structure could be recognised. Sixty hours into the rooting process a root primordium was visible, under transverse section of the hypocotyl the primordium was circular and approximately 150-200 cells could be seen in the largest cross section (Plate 4.1d). The root primordium then elongated (Plate 4.1e), with the first visible roots emerging approximately 100 hours after striking the cuttings. There was a correlation between position in the longitudinal section and root primordia formation. The root primordia all formed from cells in close proximity to the 4 vascular bundles. Even at an early stage, for example when the nuclei were pronounced, the location of the future root primordia was clear (Plate 4.2). The events described above only occurred in the rooting zone, no cell division was observed in hypocotyl sections away from the rooting zone. The rooting percentage of the cuttings was ninety three percent.

**Hormone Analysis.**

IAA concentrations in the non-rooting zone (apical 2.5mm of hypocotyl) remained relatively constant throughout the rooting process (Figure 4.9), although there was a small rise in IAA after 10 hours. A much greater increase ($P<0.02$) in IAA concentration occurred in the rooting zone 10 hours after cutting excision, although after 20 hours IAA concentrations were much lower (Figure 4.9). This transient increase in free IAA coincided with the inductive stage of ARF. The peak of IAA occurred before the first visible signs of ARF, i.e. nuclear swelling, (Plate 4.1b). At the time of the first cell divisions (Plate 4.1c), there was no significant difference in IAA concentrations between the rooting and non-rooting zone (Figure 4.9). A further

Plate 1 The anatomical sequence of events during ARF in 14-day old *E. globulus* seedling hypocotyl cuttings.

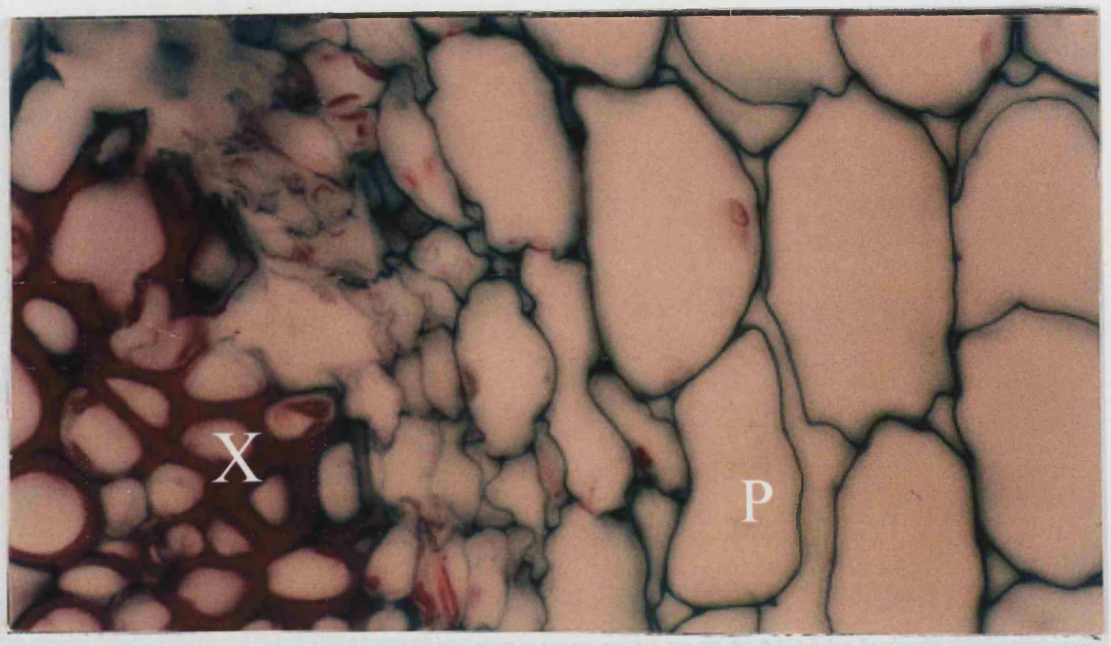
A. Pre-visible events (20 hours after cutting excision). [ x 280 Mag]

No visible cell activity.

X= xylem, P= parenchyma.

B Nuclear swelling (35 hours after cutting excision) in a few cells in the pericycle associated with the four vascular bundles. [ x 280 Mag]

N= prominent nuclei, X= xylem, P= parenchyma.


X
P

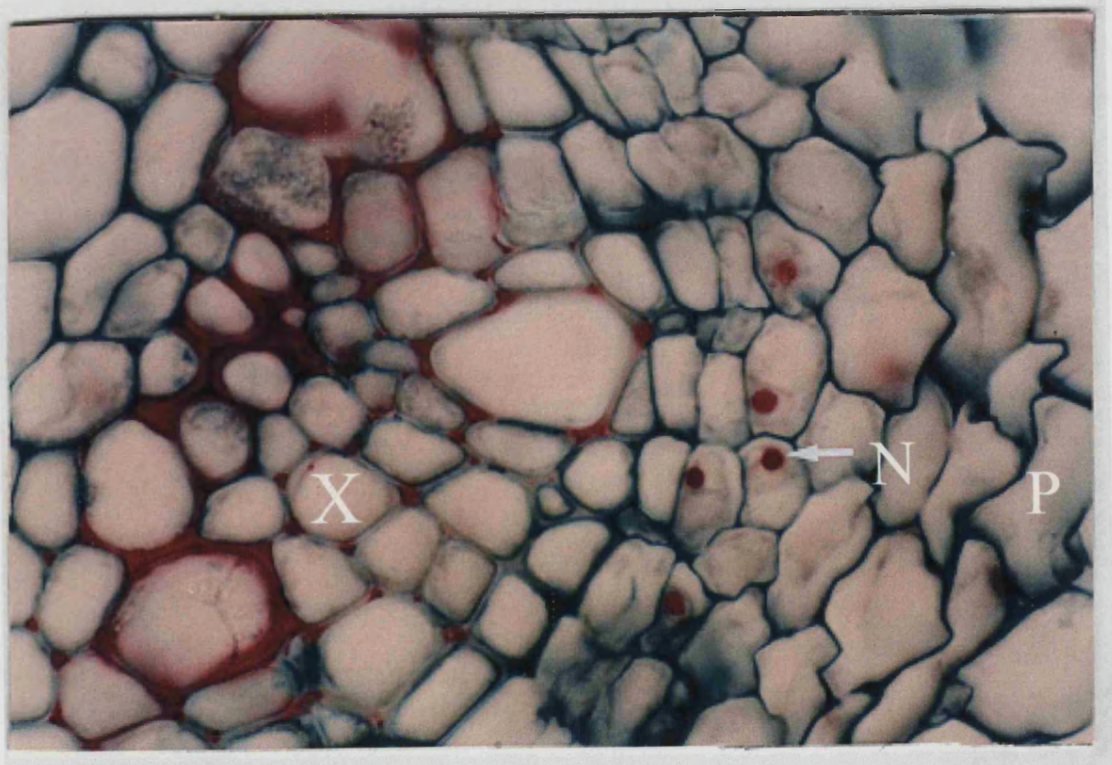

X
N
P

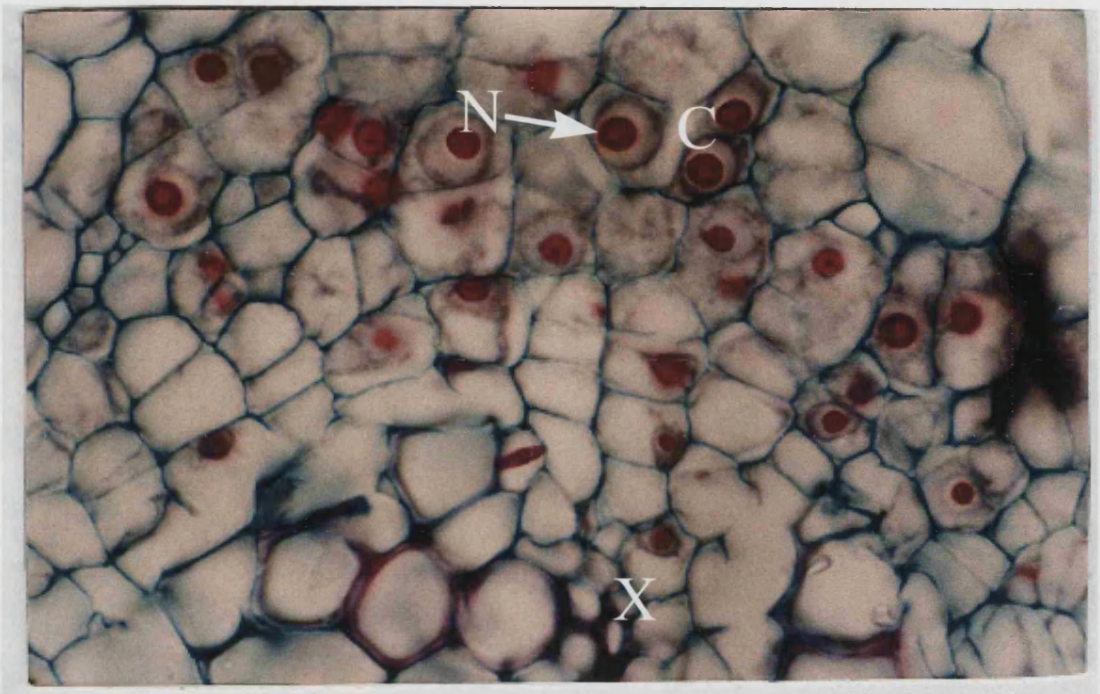
Plate 1 The anatomical sequence of events during ARF in 14-day old *E.globulus* seedling hypocotyl cuttings.

C. Early cell divisions (45 hours after cutting excision) in some cells in the pericycle in close proximity to vascular material. [ x 280 Mag]

N= prominent nuclei, C= early cell division, X= xylem

D. An organised root primordium (60 hours after cutting excision). [ x 140 Mag]

V= vascular bundle, RP= root primordium.


N
C
X

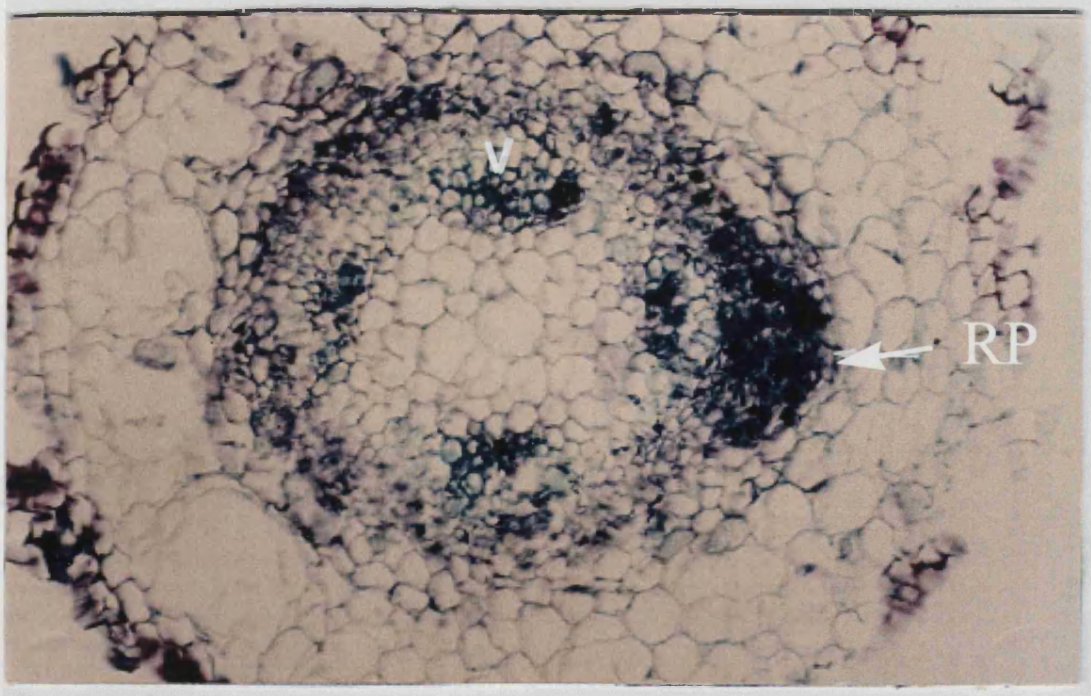

V
RP

Plate 1. The anatomical sequence of events during ARF in 14-day old *E. globulus* seedling hypocotyl cuttings.

E. Extension growth of the root primordium across the cortex (80 hours after cutting excision. [ x 280 Mag]

E= extension growth.

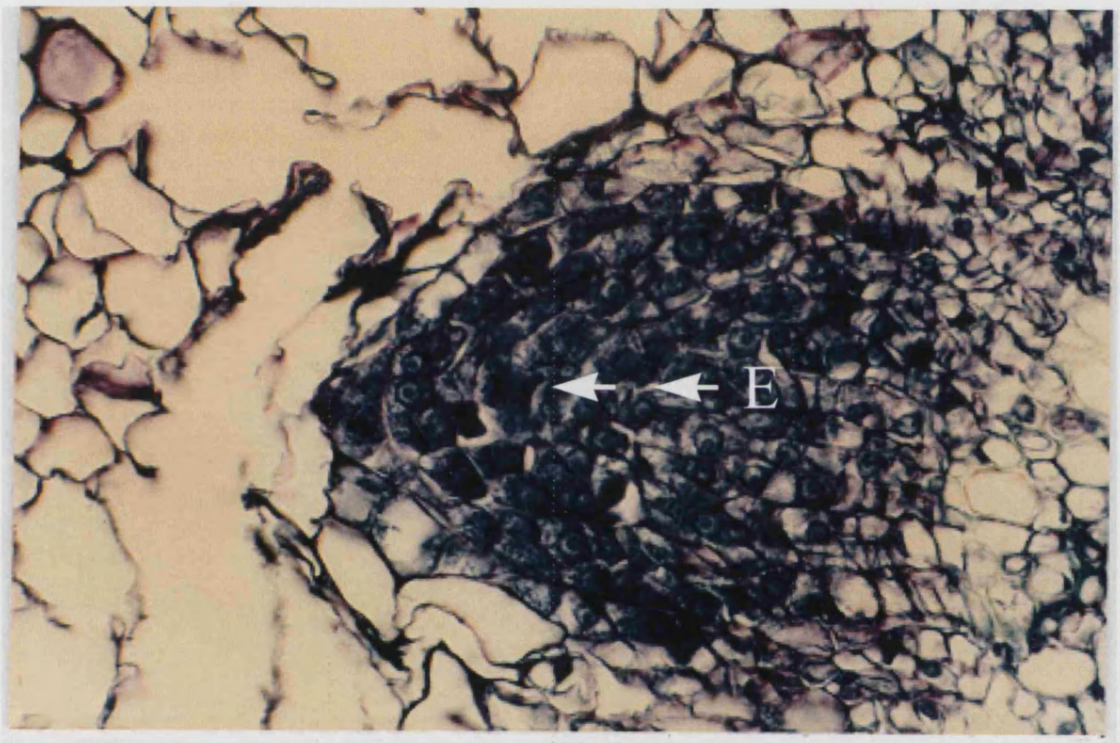

E

Plate 2. Localisation of the early anatomical events in cells closely associated with the vascular bundles during ARF in 14-day old *E. globulus seedling* hypocotyl cuttings.

E= early anatomical events (swollen prominent nuclei and early cell division),

V= vascular bundle, P= parenchyma

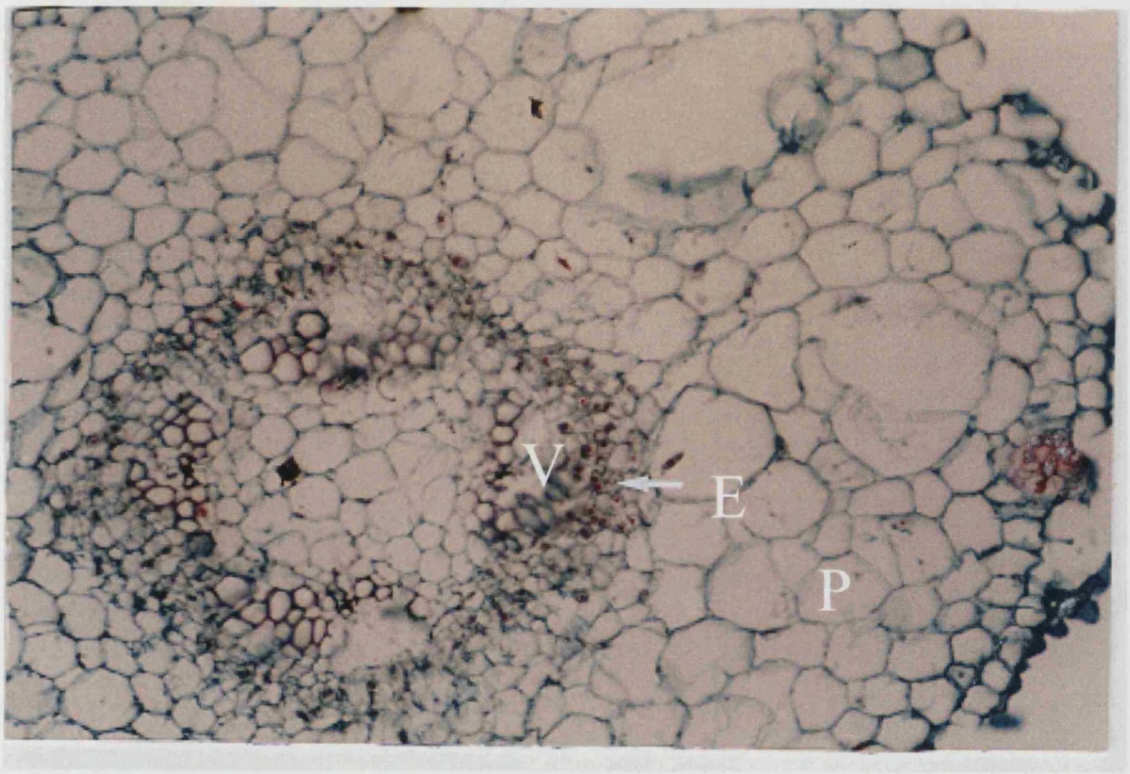
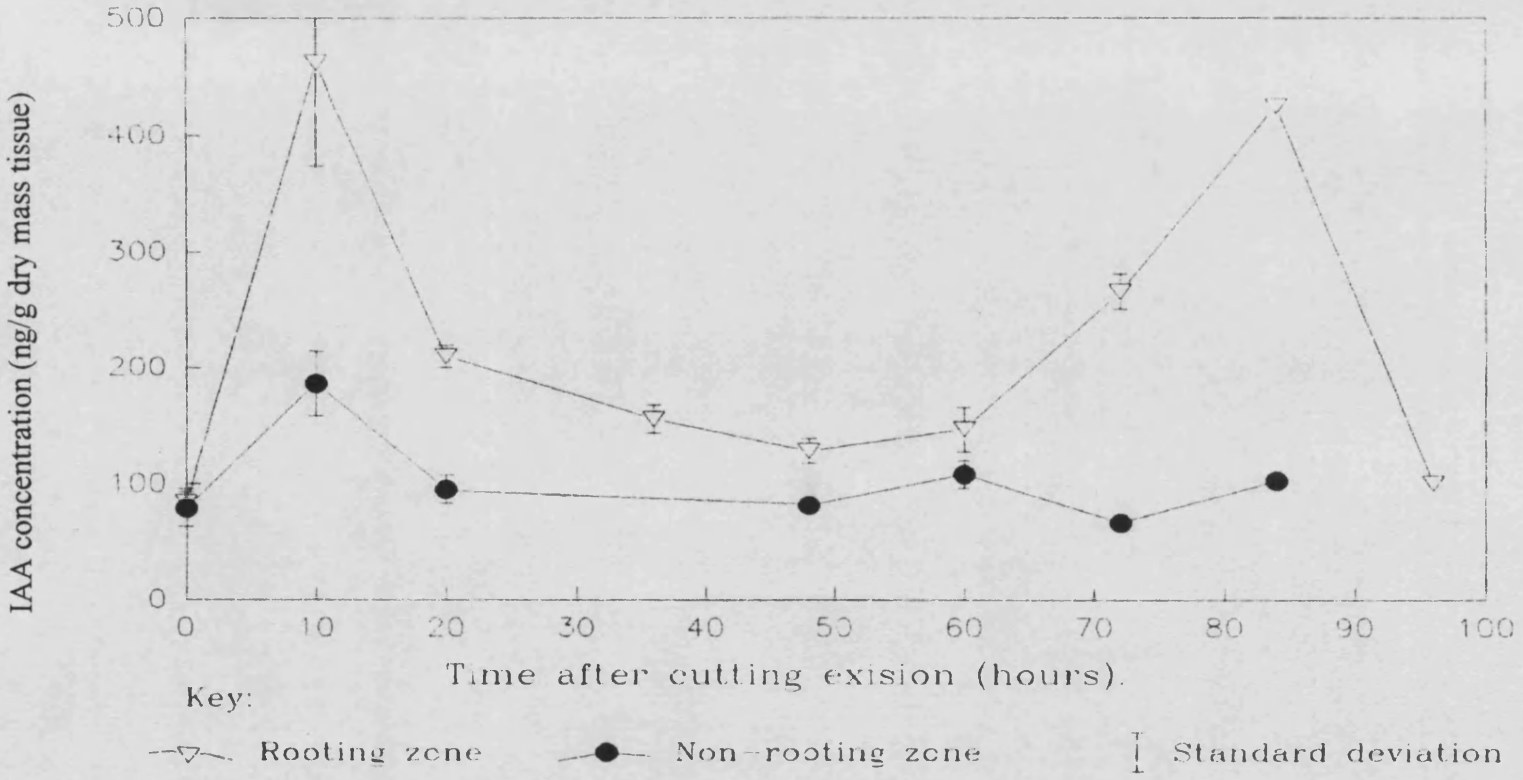

V
E
P

Figure 4.9 Endogenous IAA levels during ARF in 14 day old *in vivo E. globulus* seedling cuttings using EI-GC-MS-SIM. Two nano-grams of $^{13}C_6$-IAA was added to each sample as internal standard. Samples from the basal 2..5mm of hypocotyl (rooting zone) and the apical 2.5mm of hypocotyl (non-rooting zone) were purified using $C_{18}$-Sep-Pak and reverse phase HPLC.



rise in IAA concentration was detected in the rooting zone 72 hours after excision (Figure 4.9), which was associated with early extension growth of the root primordium (Plate 4.1e) and over the next 24 hours, during continued extension growth. The first visible roots began emerging at around 100 hours after striking the cuttings, by which time IAA concentrations were low again. The main conclusion of this experiment was that an early, transient rise in IAA concentrations occurred in the rooting zone, but not in the non-rooting zone. However, this transient rise was indicated by just a single replicated point. Consequently, it was decided necessary to repeat this experiment, incorporating more harvests over the first 24 hours.

## 4.7 Endogenous IAA concentrations during the rooting of *E. globulus* seedling cuttings.

This experiment was designed to provide a more comprehensive study of early changes in IAA concentration in particular during the first 24 hours after cutting excision and to confirm the transient increase in IAA in the rooting zone observed in experiment 4.6. Comparison of IAA concentrations in the rooting zone (basal 2.5mm of hypocotyl), with the adjacent 2.5mm (lower non-rooting zone) were made to help indicate how localised the transient increase in IAA concentration is.

The same procedure used in experiment 4.6 was followed with two modifications. Firstly more harvests were incorporated during the critical induction time. Material was harvested at 0,6,12,20,25,30,35,40,45,50,55,60 and 70 hours after cutting excision. Secondly in addition to the top 2.5mm (apical non-rooting zone) and basal 2.5mm (rooting zone), the adjacent 2.5mm above the rooting zone was used (basal non-rooting zone).

Anatomy.

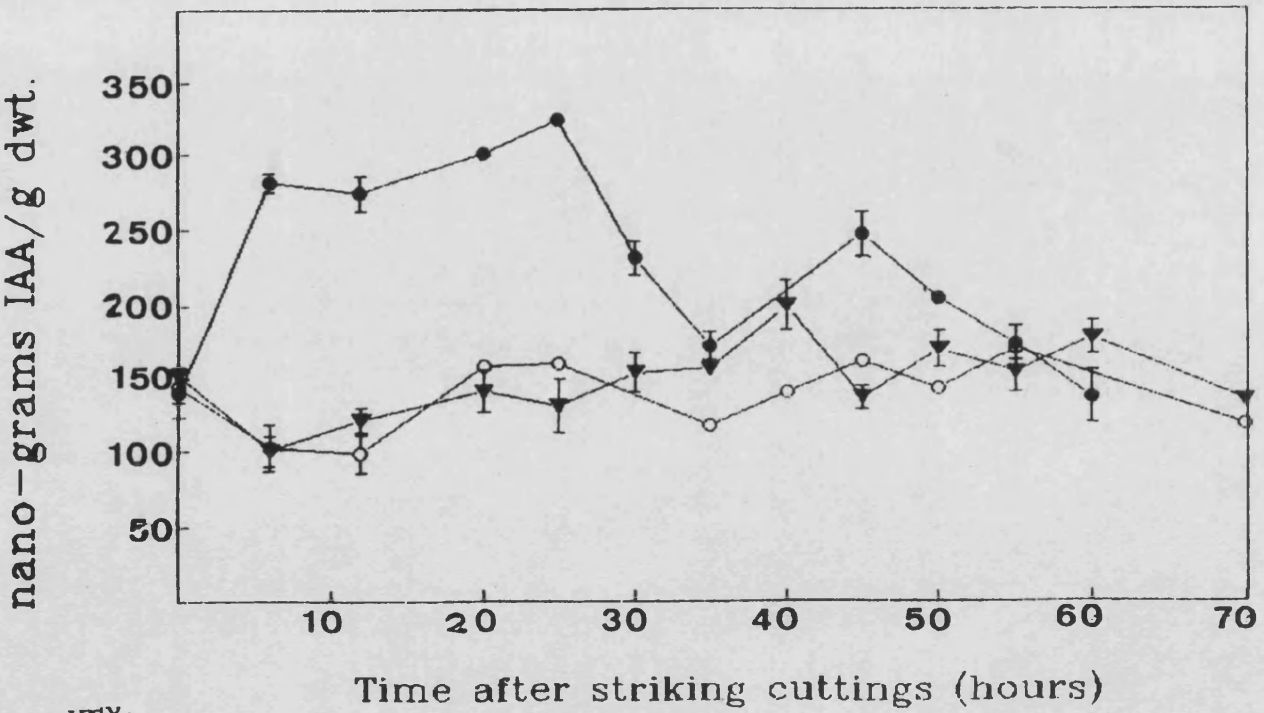
As with the previous experiment, a histological study was carried out in parallel to the analysis of endogenous IAA. This demonstrated that the timing of the various stages in root initiation were very similar between the two experiments. Thirty hours after cutting excision, nuclei of cells in the pericycle associated with the vascular bundles became swollen; this was the first visible sign of root initiation. During the next 5 hours these nuclei became more prominent. The first cell divisions took place in the cells with prominent swollen nuclei approximately 45 hours into the rooting process. A few nuclei in the cells close to the ones near the vascular bundles became prominent but did not develop any further. An organised root primordium consisting of approximately 100 cells in cross section could be seen after 60 hours. The root primordium extended across the cortex through the next 40 or so hours, the first visible roots emerging at around 100 hours after cutting excision. Outside the rooting zone (basal 2.5mm of hypocotyl) events were quite different, approximately 20 hours after cutting excision there were a few cells in the pericycle associated with

the vascular bundles whose nuclei were more prominent than the nuclei of surrounding cells, but this was a rare event and increased prominence and cell division did not follow on. Thus the histological sequence of events resulting in root primordia were localised to the basal 2.5mm of hypocotyl, from which the roots emerged, in these seedling cuttings.

IAA analysis.

IAA concentrations in the lower and upper non-rooting zones remained fairly constant throughout the rooting process, although there was a slight decrease during the first 12 hours in these two zones (Figure 4.10). In contrast, in the rooting zone itself a significant increase ($P<0.02$-$0.01$) in IAA concentrations occurred during the first 24 hours after cutting excision (Figure 4.10); during this period no visible signs of ARF were observed . Thirty hours after excision IAA concentrations in the rooting zone declined (Figure 4.10), as nuclei in some cells of the pericycle in close proximity to the vascular bundles became prominent. By 35 hours into ARF these prominent nuclei were even more so (Plate 4.1b), this stage was associated with low IAA concentrations. Early cell division was underway 45 hours after cutting excision accompanied by an insignificant rise in IAA concentrations (Figure 4.10). During the continued cell division which resulted in a visibly organised root primordium, and its subsequent early extension growth, there was no significant difference in IAA concentrations between the rooting and non-rooting zones (Figure 4.10). The results from this experiment confirm those from experiment 4.6(A) that early events in ARF are associated with a transient rise in endogenous IAA concentrations, and that IAA concentrations started to decrease by the time the first cell divisions were underway. There was a large difference in the pattern of IAA concentrations associated with the rooting zone (basal 2.5mm) and the adjacent 2.5mm (basal non-rooting zone), indicating that events are localised and that there is a correlation between location of histological events and the transient rise in endogenous IAA concentrations. The rooting percentage of the cuttings was ninety seven percent.

Figure 4.10 Endogenous IAA concentrations during ARF in 14 day old *E. globulus* seedling cuttings using EI-GC-MS-SIM. Two nano-grams of $^{13}C_6$-IAA was added to each sample as internal standard. Samples from the basal 2.5mm of hypocotyl (rooting zone), adjacent 2.5mm of hypocotyl (basal non-rooting zone), and apical 2.5mm of hypocotyl (apical rooting zone), were purified using $C_{18}$ Sep-Pak and reverse phase HPLC.

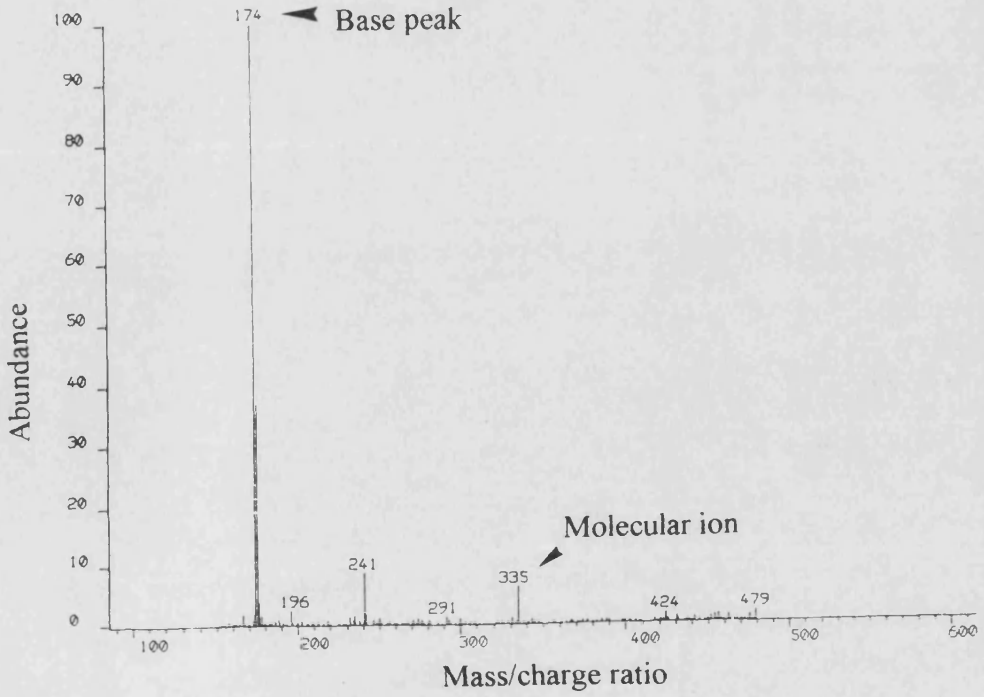


## 4.8 a Chemical ionisation GC-MS-SIM analysis of IAA.

In order to obtain lower levels of detection it was decided to employ chemical ionisation (CI) GC-MS instead of EI-GC-MS (Experiments 4.1-4.7). Negative-ion CI was used as it is a more selective process and has a lower background noise than either positive ion CI or EI, resulting in greater sensitivity. Higher sensitivity would permit smaller amounts of plant material to be used which is essential when there is a very limited amount of material available.

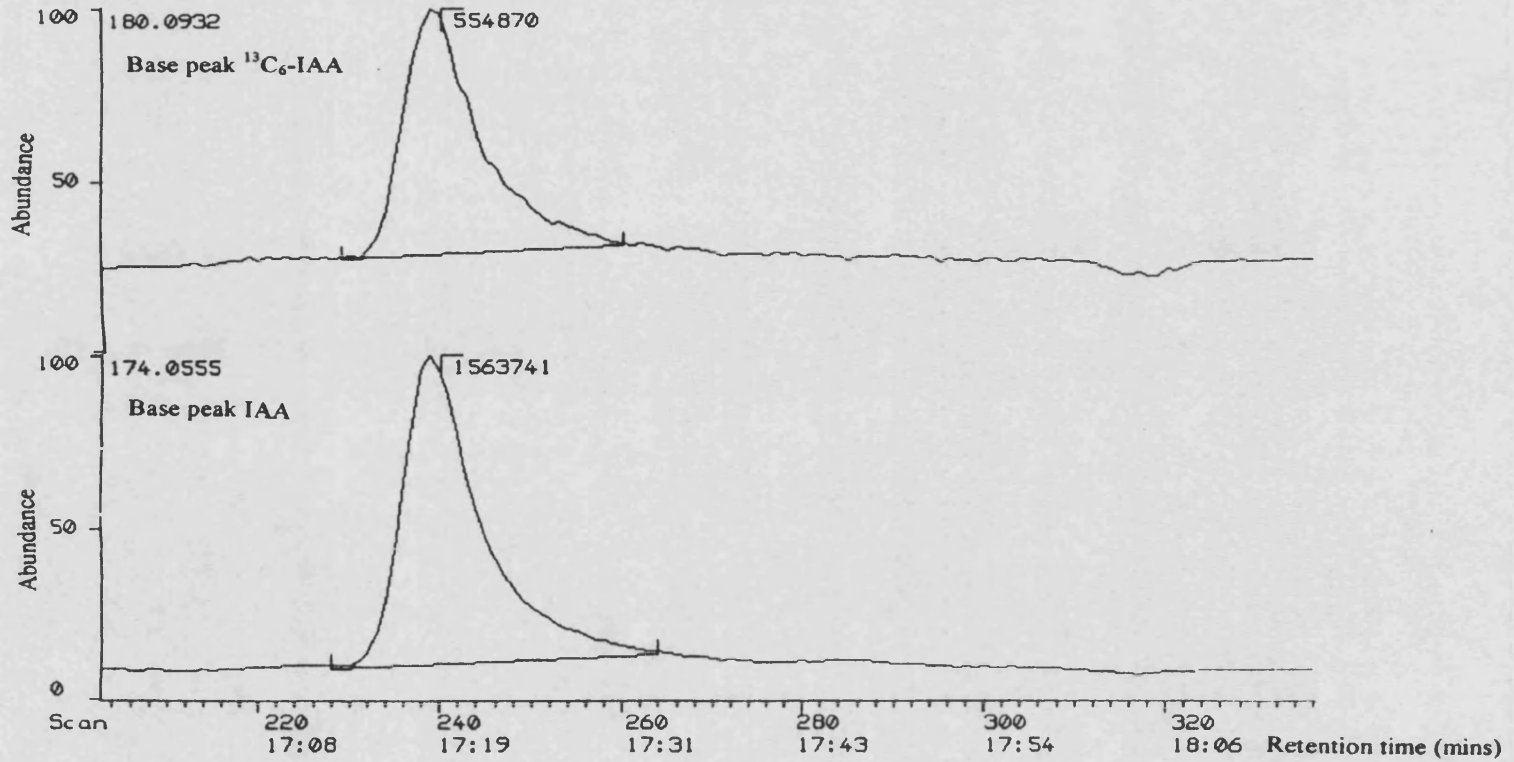
IAA solutions in acetone were derivatised using PFB (Materials and methods 2.4.3b) and run under negative-ion CI-GC-MS (Materials and methods 2.4.4ii). When in full scan mode, (Figure 4.11), the characteristic base peak (174 m/z) and the molecular peak (335 m/z) for IAA could be seen.

Figure 4.11 Mass spectrum of authentic IAA standard derivatised with PFB run under negative-ion CI-GC-MS. Sample from 10ng of authentic IAA derivatised with PFB, 1µl from 10µl injected.



Quantitative work was carried out using negative-ion CI-GC-MS in SIM mode. Under SIM mode only the base peak (174 m/z for the IAA and 180 m/z for the $^{13}C_6$-IAA internal standard) was monitored, this was done under high resolution (Figure 4.12).

Figure 4.12 The base peaks of IAA and $^{13}C_6$-IAA PFB esters from purified *in vitro E. globulus* shoot material run under negative-ion CI-GC-MS-SIM.



## 4.8 b Endogenous IAA concentrations in non-rooting *Eucalyptus globulus* seedling cuttings.

The switch to the negative-ion CI-GC-MS technique was made to obtain greater levels of sensitivity so that less material in the initial extraction was necessary. The aim of this experiment was to record endogenous IAA concentrations in 35-day old cuttings that did not root, over a similar time period to 14-day old cuttings (Experiments 4.7 & 4.8) that do, and to make a comparison between the two systems. It was decided that with non-rooting material the number of cuttings per replicate could be reduced (from 30 cuttings per replicate to 10) as the need to get a synchronised rooting event would not be a factor.

Three days prior to cutting excision the 35 day old seedlings were transferred from glasshouse conditions (Materials and methods 2.1.2) to a Saxcil cabinet running at 25°C with continuous lighting. Cuttings, prepared by severing the hypocotyl 1cm beneath the cotyledons with a sharp razor blade, were struck in vermiculite. Harvesting occurred at 0,6,12,18,24,30,35,40,50,72, and 96 hours after cutting excision.

Anatomy.

The 10 replicate hypocotyls were cut with a sharp razor blade and immersed immediately in FAA, at each harvest. After dehydration and embedding, sections were stained in either Safranin-fast green or toluidine blue.

Hormone analysis.

The basal 2.5mm and adjacent 2.5mm of the hypocotyl were harvested for hormone analysis, these represented the potential 'rooting' zone and the basal non-rooting zone respectively. Three replicate batches of 10 cuttings were harvested. Material, harvested with a sharp razor blade, was immersed immediately in liquid nitrogen then freeze-dried. One ng of $^{13}C_6$-IAA per sample was added at the start of extraction. Purification utilised $C_{18}$ Sep-Pak and reverse phase HPLC using a $C_{18}$ column. Derivatization was carried out with pentafluorobenzyl bromide. Negative-ion

CI GC-MS in full scan mode was used for identification, and in SIM mode for quantification (Materials and methods 2.4.4).

No sign of root initiation, such as nuclear swelling or early cell division, was detected by hand sectioning and no roots emerged from the cuttings . Identification of IAA was confirmed by the presence of the characteristic ions, 174 m/z for IAA and 180 m/z for $^{13}C_6$-IAA (Figure 4.12). Six hours after striking the non-rooting cuttings, there was a large transient rise in IAA concentration ($P< 0.01$) associated with the cutting base (Figure 4.13) which was very similar to that in 14 day old cuttings that do initiate roots (Figures 4.9 and 4.10). Endogenous IAA concentrations in the non-rooting zone had not risen significantly 6 hours after cutting excision. IAA concentrations then remained low, with no significant difference between IAA concentration in the two zones. Some of the data at 12 and 18 hours after cutting excision was unfortunately lost during HPLC, which prevented adequate replication at the period where IAA concentrations are high in material that does initiate roots (Experiments 4.7 and 4.8) and therefore hindered interpretation.

## 4.9 Endogenous IAA concentrations during ARF in clonal *In Vitro Eucalyptus globulus* explants.

Advanced Technologies (Cambridge) Ltd. supplied 2 clonal *in vitro* lines of *E. globulus*. An 'easy-to-root' clone, was reported to root well with a 10-20 μmol IBA pulse whereas a 'difficult-to-root' clone does not root. The shoot clumps had been subcultured on a BAP-containing medium for several years.

In order to obtain explants suitable for rooting, initially the clumps were transferred for 10 days to a hormone-free medium containing 1/2-strength MS, 0.3% w/v activated charcoal (A.C.), 0.7% w/v agar and 0.06M sucrose. Magentas were used to contain the medium, and these were transferred from growth room conditions to a Gallenkamp cabinet operating at 25°C with continuous lighting (45μmol $m^{-2}$ $s^{-1}$). The clumps were then divided, producing single shoots which were selected for uniformity having 1cm of stem and healthy shoot growth. Shoots were placed onto root initiation medium which consisted of 1/4-strength MS, 10 μM IBA, 0.7% w/v agar and 0.03M sucrose. After 3 days on the root-initiation medium the remaining shoots were transferred to a hormone-free medium, consisting of 0.3% w/v AC, 1/4-strength MS, 0.7% w/v agar and 0.03M sucrose, to encourage outgrowth of root primordia. Harvesting occurred at the following times 0,10,20,30,50,72,84,96,110,120,130, and 144 hours after transferring to the root-initiation medium.

Anatomical studies.

The ten replicate explants were severed with a sharp razor blade, producing 1cm. of stem, sectioned by hand and stained with toluidine blue.

Hormone analysis.

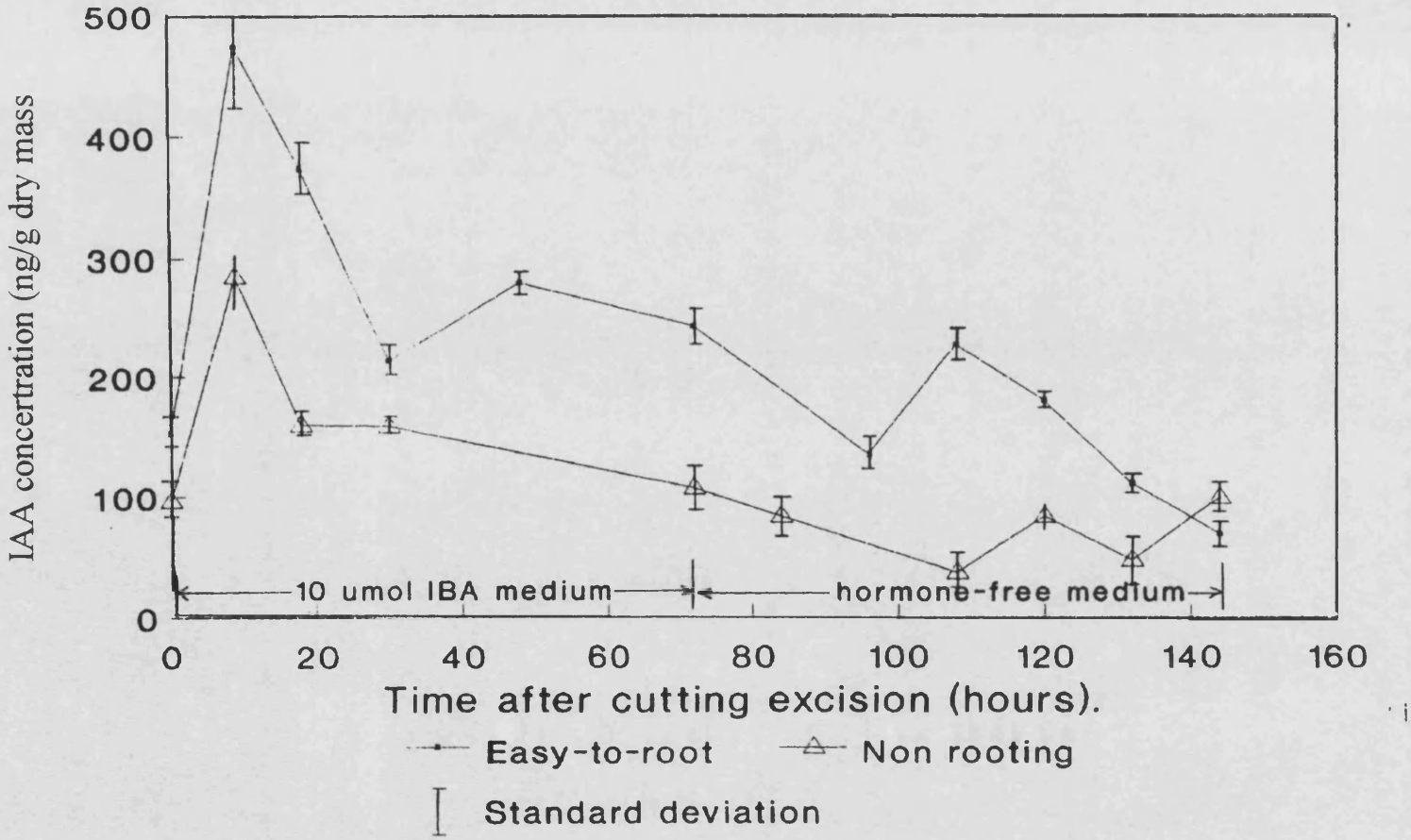
Three replicate batches of 15 explants were harvested. The basal 5 mm of stem, the approximate area over which roots emerge, was severed with a sharp razor blade and immersed immediately into liquid nitrogen then freeze-dried. The internal standard, 1ng $^{13}C_6$-IAA, was added at the start of extraction. Purification was by $C_{18}$-Sep-Pak and reverse phase HPLC using a $C_{18}$ column (Materials and methods 2.4.2iii,iv). Following purification, derivatisation was carried out using pentafluorobenzyl bromide (Materials and methods, 2.4.3). Negative-ion CI GC-MS

in full scan mode was used for identification and SIM mode used for quantification (Materials and methods, 2.4.4ii).

Results.

The rooting percentage of the 'easy', and, 'difficult'-to-root lines was 73.3% and 0% respectively. Hand sectioning showed that small round root primordia were present 80 hours after the start of the rooting protocol and that they extended across the cortex to start emerging 145 hours after the initial transfer onto the root induction medium. During the early phase (root induction), there was a large rise in free IAA concentrations in the 'easy-to-root' clone (Figure 4.14). In the 'difficult-to-root' clone during this period IAA concentrations rose, but only to about half the concentration of the 'easy-to-root' clone. The free IAA concentration in the 'easy-to-root clone' rose significantly higher ($P< 0.05$) than in the 'difficult-to-root' clone during the first 10 hours after cutting excision when the transient rise in IAA concentrations took place (Figure 4.14). In addition the peak tailed more quickly in the 'difficult-to-root' clone. Twenty hours after excising cuttings of the 'difficult-to-root' clone, IAA concentrations had fallen and continued to decline (Figure 4.14). By 30 hours IAA concentrations in the 'easy-to-root' clone had also declined considerably from the early peak (Figure 4.14). At 50 and 72 hours after cutting excision, IAA concentrations were fairly constant (Figure 4.14). Immediately after the 72 hour harvest the remaining explants were placed onto the hormone-free media containing activated charcoal. IAA concentrations remained low 96 hours onwards (Figure 4.14), corresponding with the extension growth of the root primordia across the cortex. Unfortunately due to the shortage of clonal material and lack of time to multiply it plus the absence of a suitable IBA internal standard it is impossible to deduce what is happening to the exogenous IBA i.e. if it is being converted into IAA and if so at what rate (see IAA analysis discussion).

Figure 4.14 IAA concentrations during ARF in clonal *in vitro E. globulus* explants using negative-ion chemical-ionization GC-MS-SIM Two nano-grams of $^{13}C_6$-IAA were added to each sample as internal standard, purification was by $C_{18}$-Sep-Pak and reverse phase HPLC.



## IAA analysis discussion.

It is well established that auxin is involved in the rooting process, although much of the data to support this is circumstantial. Quantification of endogenous IAA concentrations in the rooting zone of cuttings using reliable physico-chemical techniques (Sandberg *et al.*, 1987b), should be employed in order to obtain a clearer idea of the relationship between auxin and the phases of root initiation. The distinct phases of ARF are well documented (Doré 1965 and Girouard 1967a). Blakesley, (1984, *Phaseolus aureus*), Maldiney *et al.*, (1986, tomato), Moncousin (1989, grapevine) and Bouza *et al.*, (1994, *Peony*) all reported a peak in the concentration of IAA in the rooting zone associated with dedifferentiation, which is the first stage of root initiation. In each case, the rise in concentration of IAA was transient, and subsequently declined to a similar concentration to that at the time of cutting excision. Others, Berthon *et al.*, (1989, *Seqoiadendron giganteum*) and Hausmann (1993, poplar), have reported that no such transient rise occurs. Nordström and Eliasson (1991) concluded that root initiation may occur without increased IAA concentrations in the root regenerating zone in pea cuttings. With the lack of histological work and only harvesting once every 24 hours Nordström and Eliasson (1991) could easily have missed important early changes in the concentration of endogenous auxin. The timing of harvests is crucial if events are not to be missed, even in recent reports, the timing of harvests is such that early events are likely to have been overlooked (Hausmann 1993; Gomez *et al.*, 1994). Gomez *et al.*,(1994) using avocado microcuttings from 4-6-week old seedlings, reported that after 3 days on a rooting medium some nuclei appeared densely stained, this coincided

with the first harvest for analysis of endogenous IAA. With this timing, the pre-visible events (induction phase) which occur before the first harvest would have been missed. In addition the rooting process was only monitored every 3 days so there were very few data points; excluding time zero only 4 harvests occurred. When a cutting is excised, auxin might be expected to accumulate at the base as auxin is transported basipetally (Gorter, 1968). Severance of a shoot from its root system will inevitably have considerable effects on movement and localised accumulation of assimilates and plant growth regulators. It has been reported that this accumulation is aided by reduced activity of IAA oxidase/peroxidase (Chibbar *et al.,* 1979; Moncousin *et al.,* 1989; Hausman 1993). Peroxidase activity, measured in crude extracts of cuttings, generally follows a pattern which is the reverse of that of auxin concentration (Gasper *et al.,*1993). Pythoud and Buchala (1989) could not identify the typical peroxidase variation during the rooting of poplar cuttings, although this could be due to the purification of the extracts. Hausman (1993) working with *in vitro* poplar shoots concluded that peroxidase activity may participate in the regulation of free IAA. Following excision of vine cuttings, a decrease in peroxidase has been noted and this has been shown to coincide with an increase in IAA concentration (Moncousin *et al.,* 1988). The transient rise in IAA concentration subsequently declined accompanied by a reduction in peroxidase activity. Gasper (1981) however, proposed that the primary event of root initiation is characterised by a decrease in the concentration of IAA, although this was proposed before unequivocal evidence of the early accumulation of IAA had been obtained. In a more recent model, Jarvis (1986) proposed that auxin accumulation is associated with both the formation of the

meristematic locus and early cell divisions of the secondary phase. The third phase, continued cell divisions to form an organised meristem, is associated with low concentrations of endogenous IAA. From the available data there still is not a clear answer to whether dedifferentiation and the formation of a new meristematic locus and the early cell divisions both require an elevated concentration of IAA. Moncousin *et al.*, (1989) speculated that the enhanced IAA concentration was responsible for cell reactivation in the interfasicular cambium. In the present study, the transient rise in endogenous IAA concentrations in *E. globulus* seedling cuttings declined before the first cell divisions occurred (Experiments 4.6 and 4.7). The exact stage of the IAA concentrations decline is not clear as IAA concentrations declined before any sign of increased nuclear prominence in hypocotyl cuttings from 12 day old seedlings (Experiment 4.6). However, as there was a 15 hour time interval between the third and fourth harvest, the pattern of events is not precise enough to pin-point the exact timings. With the hypocotyl cuttings from 12 day old seedlings used in experiment 4.7 the timing of harvests was more regular, IAA concentrations were still fairly high when nuclei in some cells of the pericycle in close proximity to the vascular bundles become prominent (30 hours after cutting excision), but had decreased before the first cell divisions which occurred approximately 45 hours after cutting excision (Figure 4.10). Many workers have studied the cytological and biochemical changes associated with the early cell divisions in root formation, primarily with herbaceous cuttings. The change in pattern of IAA concentration in the rooting zone of *E. globulus* hypocotyl cuttings is similar to that reported with *Phaseolus aureus* hypocotyl cuttings where a transient rise in IAA concentration occurred prior to early

visible events only in the basal section of the hypocotyls where the roots form (Blakesley *et al.,* 1985). The first visible signs of root initiation in *P. aureus* hypocotyl cuttings are the cytoplasm and nucleus becoming dense. This occurs within the first 24 hours, with cell division following within the next 48 hours (Chang and Chan 1976). A subsequent increase in RNA synthesis which would correspond to the first cell divisions has also been demonstrated (Lee *et al.,* 1978). Both of these studies suggested that the increased synthesis of RNA could be caused by an increased auxin concentration. It is evident from the literature that a wide array of factors can influence the rooting process, although auxin has long been regarded as a central factor in this process. Improvements in techniques, including the availability of suitable internal standards, has meant that quantification of more than one substance is now easier. For example, IAA and ABA have been in the same sample (Li *et al.*, 1992; Noiton *et al.*, 1992a,b). The reports by Noiton *et al.* (1992a,b) only considered growth regulator concentrations after each subculture, i.e. there was no indication as to the importance of the substances at different stages of the rooting process. The initial nodal explants were referred to as culture 0 and shoots resulting from the growth of these buds were called subculture 1. The new shoots from subculture 1 were designated subculture 2 and so forth. Subcultures 1,2,3,4,9 and 26 were used as microcuttings, the rooting ability was poor in the early subcultures and had increased to 100% by the 9th subculture. After establishment *in vitro* there was no variation in the IAA content throughout the subcultures but a decrease in ABA content was observed after the fourth harvest, where the rooting ability increased significantly. However there was no report of a significant decrease in ABA

concentration at subculture 2, where the rooting percentage rose from 0 to 32%. The results are difficult to interpret as the growth regulator concentrations are only given for one time period rather than throughout the rooting process.

The work reported in this thesis on endogenous IAA concentrations during ARF in *E. globulus* cuttings, supports the earlier work that a transient peak in IAA concentration is associated with the dedifferentiation and formation of a new meristematic locus. An exception was in the non-rooting seedling cuttings (Experiment 4.8b), where there was a significant ($P<0.01$) transient rise in free IAA concentration in the cutting base 6 hours after cutting excision; this peak however was only represented by one point. With the loss of samples preventing adequate replication, particularly at 10 and 18 hours after cutting excision, conclusions are difficult to draw from this experiment. The actual severance could cause movement and localised accumulation of assimilates and plant growth regulators towards the base of the cutting. The difficult-to-root clone used in this study failed to root on hormone-free medium (1/4 strength MS, 0.03 M sucrose, solidified with 0.7% w/v agar) and less than 10% of the easy-to-root clone rooted on this medium. When placed on the 10 μM IBA root induction medium 73.3% of the easy-to-root clone formed roots, whereas none of the difficult-to-root clone rooted. During ARF in clonal *in vitro E. globulus* explants (Experiment 4.9), there was a transient rise in free IAA concentration during the early stages of the rooting process. Although 10 hours after transfer to the root induction medium IAA concentrations also rose in the 'difficult-to-root' clone, the concentrations were significantly higher ($P< 0.05$) in the 'easy-to-root' clone. The increased IAA

concentrations are unlikely to be due to the basipetal transport and accumulation as the micropropagule was not severed in anyway, rooting was induced by transferral from the hormone-free medium to the root induction medium (Experiment 4.9). The results are not easy to interpret with respect to the origin of the elevated free IAA concentrations. Two sources of the IAA are endogenous, from within the shoot and via conversion from IBA may be responsible for the elevated concentrations of IAA. It would have been useful to have included a control medium without IBA, but with a small amount of available material and no time left to multiply it the present system was used. In the absence of exogenous auxin it would have been possible to see whether the increase in IAA concentration is predominantly exogenous or endogenous in origin. If a suitable IBA internal standard, such as [4-$^{3}$ H] IBA had been available it would have been possible to have deduced whether or not IBA was converted into IAA in *E. globulus* tissue, and if so the percentage and rate at which it was converted. Conversion of IBA into IAA has been reported by several workers (Epstein and Lavee 1984; Alvarez *et al.,* 1989a; Van der Krieken *et al.,* 1992a,b; 1993). Van der Krieken *et al.,* (1993), working on the conversion of IBA into IAA on root regeneration in apple, reported that due to conversion of absorbed IBA into IAA a fraction of 0.4% was recovered as free IAA. They found that at equimolar concentrations, the uptake of IBA is four times higher than that of IAA so that the application of IBA led to a four-fold higher internal IAA content than application of IAA. Conversion of IBA into IAA in the test system for root regeneration in apple (Van der Krieken *et al.,* 1993) could be detected after a few hours (Van der Krieken, Personal communication). Nordstrom *et al.,* (1991) however, have reported that IBA

exerted its own effect. Experiment 4.9 nevertheless did show a positive correlation between the ease of rooting and auxin concentration, although further work is necessary to confirm this. Studies with *Hibiscus* (Bose *et al.,* 1973) and *Vitis* (Kracke *et al.,* 1981; Bartolini *et al.,* 1986) also reported a positive correlation between the ease of rooting and auxin concentration. Bouza *et al.,* (1994), reported that *Peony* explants differed in their rooting capacity depending on the origin and subculture duration and that there was a positive correlation between rooting capacity and endogenous auxin concentration. A positive correlation between auxin concentrations in the rooting ability of M9 and M26 apple rootstocks has also been found (Alvarez *et al.,* 1989a). Significantly higher IAA concentrations in the 'easy-to-root' clone (M26) were only found in the bases of the cuttings, in the apical sections of both lines free IAA concentrations were comparable. However, this is in contrast to work with apple rootstocks M26 and A2 (Welander and Snygg 1987), and with work with "Johnothan" apple (Noiton *et al.,* 1992b) in which different rooting ability was generated by the number of subcultures *in vitro.* In the latter study, IAA concentrations were only monitored at each subculture, no quantification of IAA concentrations was made during the rooting process. The work in experiment 4.9 shows that there is a significant difference ($P<0.05$) in free IAA concentrations between the two clones at time zero, and IAA concentrations were significantly higher ($P<0.05$) in the 'easy-to-root' clone 10 hours after transferring to the root induction medium.

For the purpose of this work competence is defined as the ability of cells within tissues to respond to specific root-inducing stimuli by the

formation of roots. Once competent cells/tissues have been exposed to an inducer the tissues may become determined for root formation. Determination is defined as the commitment of cells to a specific developmental fate (Meins and Binns, 1979). Thus, once a cell, or group of cells, has received a signal for root formation, they will remain committed to root formation even upon the removal of the signal (Mohnen 1994). The state of determination can be ascertained by experimental manipulation of cells, tissues or organs (Meins and Binns, 1979). Frequently the experimental manipulation used for studies of determination for root formation has involved transfer of tissue explants from media containing a root-inducing factor, so-called root inducing medium (RIM) to a medium without the factor (basal medium) and studying root formation after a fixed period of time (Mohnen 1994). Tissues are determined for root formation at the point in time when, following removal from the root-inducing factor, they continue with root formation. The root-inducing factor used in experiment 4.9 was 10 μM IBA, and the explants were exposed to this for 3 days before being transferred to hormone-free medium. Of the two clones used, 73.3% of explants from the easy-to-root clone formed roots whereas no explants from the difficult-to-root clone rooted. With more time and more clonal material it would have been useful to include a control medium without IBA and also to vary the exposure time of the easy-to-root clone to find out the time it takes for the explants to become determined. Earlier experiments with difficult-to-root clones of *E. globulus* have identified material that failed to root in response to a wide range of applied auxin concentrations (A. Brackpool, Personal Communication). It is not clear whether the difficult-to-root clone fails to reach a state of competence or of determination.

In addition to higher free IAA concentrations being associated with higher rooting ability, Alvarez *et al.,* (1989a) also found a greater proportion of IAA was present as a conjugate in the difficult-to-root shoots. IAA conjugates have no auxin activity *per se*, and their activity is directly related to the amount of free auxin released by hydrolysis (Bialek *et al.,* 1983). IAA conjugation is usually regarded as a reversible process and the conjugate as a potential source of free IAA (Bialek and Cohen 1989). The experiments in this thesis did not involve work on conjugates so their importance in the rooting process is not known for the *E. globulus* material used , also the source of the transient rise in free IAA concentrations is not known, i.e. the involvement of auxin synthesis and hydrolysis of auxin conjugates was not studied. A number of workers have implicated auxin conjugates in the control of ARF. Blakesley *et al.,* (1991b) examined the concentrations of IAA and IAA conjugates in cuttings of *Cotinus coggyria* taken at different times of the year. Cuttings taken at the time of bud break rooted well, but cuttings taken much later in the growing season rooted very poorly. At the time of harvest, free IAA concentrations in young shoots were significantly higher than those of IAA conjugates. Later in the season, when rooting was poor, the reverse was found. Although the "pool" of IAA was similar on both occasions, the ratio of free IAA to total IAA in the rooting zone was 0.94 in young shoots which rooted, and 0.02 in older shoots which failed to root.

The work carried out in this thesis has specifically concentrated on absolute concentrations of IAA during ARF. Trewavas (1981) brought to the fore the theory that in many cases sensitivity (responsiveness) to plant growth substances is more important than absolute concentrations of plant growth

substances, and has discussed the actual measurement and theoretical basis of sensitivity (Trewavas 1991). His criteria for an unambiguous measurement of growth substance sensitivity are not easy to meet due to the experimental constraints, but would permit the measurement of sensitivity at the plant growth substance concentration which Trewavas (1991) defined as control strength. Control strength is difficult to apply to the plant tissue that either roots or does not root, but could prove useful when considering increased root numbers with increased auxin concentrations. Sensitivity to plant growth substances is difficult to measure, partly because of the problems in meeting the criteria for an unambiguous measurement as defined by Trewavas (1991). Virulent *A. rhizogenes* and *A. tumefaciens* bacteria harbour a Ri (root inducing) plasmid and a Ti (tumour inducing) plasmid respectively. These plasmids are of interest as they contain genes involved in the biosynthesis of plant growth substances. In addition transfer of certain regions of the Ri plasmid has been reported to confer increased sensitivity to auxin in plant tissue (Maurel *et al.,* 1991; Shen *et al.,* 1988). The apical region of 7 week old pRi transformed and *rol*-A tobacco plants contained about half the endogenous IAA concentration present in the apical region of control plants, along with an attenuated basipetal auxin gradient (Prinsen *et al.,* 1994). Concomitant with the reduced IAA concentrations, no transient ABA accumulation was observed in transgenic shoot apices. In addition to the elevated IAA concentrations found in 6-12 week old normal shoot tips, an elevated cytokinin content was reported at the beginning of this period which corresponded to a mature developmental stage where competence for flowering is acquired (Prinsen *et al.,* 1994). The detailed kinetic hormone analyses (Prinsen *et al.*, 1994) emphasised the relevance of

varying hormone concentrations in the shoot apical region in the developmental pattern of both transgenic and normal plants. *Rol* gene loci have been studied by many groups, Shen *et al.,* (1988), Maurel *et al.,* (1991), Schmulling *et al.,* (1993). Work on *rol* genes could prove a very useful tool in enhancing our knowledge of the role of plant growth substances in the process of root initiation. They may permit the manipulation of endogenous IAA concentrations in the rooting zone through auxin biosynthesis (Blakesley, 1994). Maurel *et al.,* (1991) postulated that increased sensitivity to auxin could be a major determinant for root differentiation and indeed that the increased sensitivity to auxin conferred by the *rol*B gene might direct the transformed cell into root organogenesis. The importance of sensitivity to auxin in relation to ARF is not understood and there is little work on it in the literature. There is considerable evidence documenting a peak in the concentration of IAA in the rooting zone associated with dedifferentiation and the formation of a new meristematic locus (Blakesley 1984; Maldiney *et al.,* 1986; Moncousin *et al.,* 1988; Gaspar *et al.,* 1990).

The techniques for analysis of absolute concentrations of plant growth regulators have improved considerably in recent years. The availability of suitable stable isotope labelled 'heavy' internal standards such as $^{13}C_6$-IAA (Cohen *et al.,* 1986) and a new generation of comparatively low cost bench-top GC-MS instruments becoming available (Rivier 1986), has given researchers access to reliable, accurate methods of quantification. Another technique which is available for increased sensitivity is the use of CI-GC-MS, which can result in 5 to 10 fold ion responses compared with the EI mode of fragmentation (Rivier and Saugy 1986). As negative-ion CI-GC-MS is also a more selective

process and has a lower background noise than either positive CI or EI, even if the intensity of the signal is not greater, there is likely to be a 20- to 50-fold increase in sensitivity (Rivier and Crozier 1987). Negative-ion CI-GC-MS was used for quantification of endogenous IAA concentrations in non-rooting cuttings (Experiment 4.9b) as the more sensitive technique permitted a smaller number of cuttings to be used, thereby saving time in the preparation and harvesting of cuttings. When endogenous IAA concentrations were quantified in clonal *in vitro E. globulus* explants (Experiment 4.9), the negative-ion CI-GC-MS technique was used for two reasons, firstly there was a very limited amount of clonal material available so the enhanced detection limits permitted less material to be used. The *in vitro* conditions resulted in more time being spent in preparing and harvesting cuttings, so the technique saved a lot of time as fewer explants were necessary using the more sensitive technique. Negative-ion spectra tend not to be used that frequently as they generally contain fewer fragments and as a result are less informative for qualitative analysis (Sandberg *et al.,* 1987a). In the present work the negative-ion technique was used for quantitative analysis, there was little background noise due at least in part because negative-ion CI is a more selective process than either positive CI or EI. There is still very little work employing the use of negative ion GC-MS. The improved concentrations of detection permit much smaller pieces of plant material to be analysed, so a clearer picture of the IAA concentrations in the region that initiates the rooting process should be easier to achieve. Epstein and Cohen (1981), using negative-ion chemical ionisation GC-MS-SIM with ammonia as the reagent gas obtained detection of 5 pg of IAA as its PFB ester.

With young seedling cuttings (Experiments 4.6 and 4.7) and the *in vitro* explants (Experiment 4.9) I believe we have virtually reached the practical limits of detection for endogenous IAA. Taking the young seedling cuttings (Experiments 4.6 and 4.7) for example, harvesting the basal 2mm of hypocotyl could not be reduced by much without the risk of missing the events as root initiation occurs along this 2mm length. In addition, in order to obtain a synchronised series of events it is not possible to vastly reduce the number of cuttings. Although the primary events with these cuttings were found to occur in a few cells of the pericycle associated with the four vascular bundles, it is not practical to measure IAA concentrations in this area.

Immunocytochemical localisation and photoaffinity labelling are two techniques that could be developed to aid our understanding of the role of IAA in ARF, possible application of the two techniques are described in the general discussion under future work.

# Chapter 5. General discussion.

In chapter 4 the central role of IAA in the rooting process was discussed. Whilst auxin is generally accepted as playing a central role in the rooting process, which is supported by a wide array of direct and indirect evidence, many other factors have also been reported to have an effect on the rooting process (Haissig, 1986; Jarvis, 1986). Other factors which can effect the rooting process include other plant growth regulators, such as, cytokinins (Maldiney *et al.,* 1986; Anderson and Camper, 1987; Bollmark *et al.,* 1988; Label *et al.,* 1988; van Standen and Hartney, 1988; Bouza *et al.,* 1994), abscisic acid (Label *et al.,* 1988, 1989; Berthon *et al.,* Blakesley *et al.,* 1991b; Noiton, *et al.,* 1992), ethylene (Robbins *et al.,* 1983; Mudge, 1988; Moncousin *et al.,* 1989; Riov and Yang, 1989; Liu and Reid, 1992) and gibberellins (Fabijan *et al.,* 1981). Carbohydrates have been shown to be able to affect the rooting process (Jarvis and Booth, 1981; Tran *et al.,* 1985; Veieskov, 1988) as have genetic effects (Locy, 1983; Haissig *et al.,*1992) and a wide array of environmental factors (Haissig, 1986; Loach, 1988).

The histological work on the rooting of young *E. globulus* seedling cuttings presented here fits in with the general view that ARF consists of four distinct phases. These essentially include:

1. Pre-cell division, where differentiation and the formation of a new meristematic locus occurs.
2. Early cell divisions producing a cluster of cells which are radially symmetrical.
3. Later cell divisions to form a discernible root meristem.
4. Root formation by extension growth of cells produced by the meristem.

(Doré, 1965; Girouard, 1967a).

The present work is in agreement with that of several others who worked with easy-to-root cuttings and reported that there was a transient rise in free IAA

concentrations associated with the induction period of ARF, followed by a subsequent decline in auxin concentrations which coincided with early cytological events (Blakesley, 1984; Moncousin *et al.,* 1989; Gaspar *et al.,* 1990). In the young easy-to-root seedling material there was a positive correlation between the site of ARF and the localisation of elevated IAA concentrations. IAA concentrations in the non-rooting zones remained fairly constant throughout the rooting process. In contrast, in the rooting zone itself, a significant increase in IAA concentrations occurred during the first 24 hours after cutting excision. This early transient rise in IAA concentration which only occurs in the basal region of the cuttings was also reported by Blakesley *et al.,* (1984) working with *Phaseolus aureus* hypocotyl cuttings and by Moncousin *et al.,* (1989) working with an *in vitro* grapevine. There are some conflicting reports, especially with the earlier work for example Bose *et al.,* (1973) and Weigel *et al.,* (1984); however frequently such work either is reliant on procedures that do not employ sound physico-chemical techniques, or, the IAA analyses are timed such that crucial early stages could very easily have been missed.

In the present work with clonal explants, IAA concentrations in the easy-to-root clone (73% of explants rooted) were significantly ($p< 0.05$) higher than in the difficult-to-root clone (0% of explants rooted) at time zero. IAA concentrations in the difficult-to-root clone during the first 10 hours after cutting excision rose, but only to about half the concentration that occurred in the easy-to-root clone. The free IAA concentration in the easy-to-root clone rose significantly higher ($P<0.05$) than in the difficult-to-root clone during the first 10 hours following cutting excision when the transient rise in IAA concentration took place. In addition the peak tailed more quickly in the difficult-to-root clone. As the root-induction medium contained 10 μM IBA and there was no suitable internal standard available, the source of the elevated IAA concentration is not known. The elevated IAA concentration could be due to conversion of IBA into IAA (Epstein

and Lavee, 1984; van der Krieken *et al.,* 1993), or endogenous, or a combination of the two. It would be useful to repeat the experiment, with particular regard to the first 24 hours following cutting excision, incorporating the use of a suitable IBA internal standard, such as [4-$^3$ H] IBA, and a hormone-free control medium. This could give a clearer answer to questions such as what is the source of the increased IAA concentration and if conversion of IBA into IAA does occur, at what rate does this take place. It may also provide a clearer idea of differences between the rooting ability of the two clones in relation to auxin, for example is there a difference in the ability to convert IBA into IAA between the difficult-to-root clone and the easy-to-root clone.

IAA plays an important role in ARF, especially during the early events in the rooting process (experiments 3.6 and 3.7). Such information can be of practical importance when one is developing a rooting system for young material, for example the need for a suitable auxin pulse after cutting excision. The work in this thesis also shows that the rooting ability can be restored in material that does not root, experiment 3.4. However, the ability of auxin to restore rooting ability as the stock plants age is limited as it only delayed the age-related decline (experiment 3.5).

Maturation is clearly a complex process and while the sharp decline in rooting ability is taking place many changes are occurring for example, leaf shape changed from being ovate to lanceolate , and the stem morphology changed from square in cross section into circular. There is very little work in the literature on the relationship between the onset of maturation and endogenous IAA concentrations. It is clear that endogenous IAA concentrations do not entirely explain the decline in rooting ability during maturation, but particularly in the early stages of maturation, IAA concentration could be important, as suggested by the indirect evidence involving application of IBA and the subsequent increase in rooting percentage. An attempt was made in this study to quantify endogenous IAA concentrations in non-rooting cuttings (experiment 4.8b), to

see if IAA concentrations changed in older material that is unable to root without auxin application. Although there was a significant ($P<0.01$) transient rise in IAA concentration in the cutting base 6 hours after cutting excision, the loss of samples, particularly at 12 and 18 hours after cutting excision, resulted in this being a single replicated data point and therefore impossible to draw conclusions from. The experiment should be repeated to see if there is a peak in IAA concentration, with particular regard to the first 24 hours following cutting excision. If there is a peak in IAA concentrations following cutting excision in non-rooting cuttings, careful interpretation will be required. One consideration would be the responsiveness of the material to the IAA and the distribution within the tissue. Using *in vitro* material, where the explants were transferred to root induction medium (containing 10 μM IBA) and utilising a suitable IBA standard to monitor whether IBA is converted into IAA, and if so at what rate, should help answer the question of whether the actual severance to produce the cutting contributes to the elevated IAA concentrations in the cutting base, as this too could be a factor in the non-rooting material.

There is also a need for a detailed study on IAA concentrations during the decline in rooting ability with increasing age of woody species, *E. globulus* with its rapid decline in rooting ability is a good candidate for such work.

Future Work.

Future directions in the study of ARF have been covered by many workers (Davis and Haissig 1994). Areas that are gaining interest include the search to find markers for juvenility concentration that will allow successful propagation (Franclet *et al.,* 1987). At present there is no direct evidence linking a biochemical or molecular event for competence to root, the accumulated evidence is largely correlative (Hand, 1994). Progress has been made on identification of at least some members of the consortium of genes whose transcription imparts the necessary and sufficient conditions

for rooting (Blakesley and Chaldecott 1993). Studies of pre- and post-transcriptional factors that regulate expression of identified genes can now be followed by using structural and promoter gene sequences as molecular probes. Most of the plant cell lines that have been developed are cell suspension or callus lines, and are quite heterogeneous in cell composition, as determined by monitoring cellular metabolism and response of the cells to exogenously supplied factors (King *et al.,* 1973). The requirements for establishing homogenous plant cell lines are complex to meet (Ernst 1994). However, the homogenous system could prove useful in studying the effects of specific factors on cellular differentiation, including cell-types that make a *de novo* meristem at various stages of development. It is generally accepted that lateral root formation occurs in the pericycle adjacent to the vascular poles (Charlton 1991). Ernst (1994), suggested the possibility of developing a homogenous culture of pericycle cells that retain enough *in vivo* character so that some of the causal factors associated with competence changes in lateral root induction could be studied in isolation. The production of homologous plant cell lines could also prove useful in a better understanding of factors effecting the early stages of ARF.

Immunocytolocalization techniques could be employed to aid our understanding of the role of IAA in ARF. The technique enables the localisation of plant growth regulators in tissues and cells using antiplant growth regulator antibodies, which are subsequently detected using anti-serum to the primary antibody linked to the antigen. These can be visualised using light or electron microscopy. Immunocytolocalisation has been used in studies on IAA by a variety of workers including Ohmiya *et al.,* (1990), who localised IAA and found that subcellular localisation is tissue specific in peach seedlings. In the same study they also demonstrated that IAA accumulated in the nucleoli of meristematic cells of the root tip. Ohmiya and Hayashi (1992) looked at subcellular localisation of IAA in leaf cells of *Prunus persica* at different stages of

development. The study showed that IAA was distributed throughout the cytoplasm, nuclei and cell wall, and that the distribution pattern changed as cells matured. One important application of immunocytolocalisation in the present work on ARF would be to provide information on the distribution of IAA particularly in the first 24 hours following cutting excision. In experiments 4.6 and 4.7 a few cells in the pericycle associated with the four vascular bundles became noticeably swollen and prominent, this was the primary visible event in ARF. By investigating localisation and accumulation of IAA in this system, a clearer picture should arise about why these cells respond with regard to auxin. One such example is that it could be that as these cells are located in close proximity to the vascular bundles they are the first to receive the auxin stimulus and therefor the first to respond. Another possibility is that IAA accumulates in the cells of the pericycle in close proximity to the vascular bundles, this accumulation of IAA triggering the activity in these cells. In this situation other cells in the pericycle could be unable to respond as the necessary IAA is used by the cells in closer proximity to the vascular bundles. To utilise immunocytolocalisation for studying IAA in *E. globulus* material, careful checks would be necessary as several problems could arise. Firstly there must be no or negligible redistribution of IAA. As IAA is soluble in organic solvents and water, redistribution of IAA during tissue preparation for microscopy is important to overcome. Another important potential problem to overcome is cross-reactivity, monoclonal antibodies with a high specificity are important where structurally similar indoles and IAA conjugates are present. This technique was used by Shi *et al.*, (1993) in the primary roots of *Zea mays*, and was the first study to localise IAA in dicotysomes and dicotysome-derived vesicles, and indicated that dicotysomes and vesicles constitute a pathway for IAA movement in and secretion from root cap cells. The use of this new technique should enhance our understanding of the role of plant growth regulators in plant growth and development.

Photoaffinity labelling is another microtechnique that could improve our understanding of the role of IAA in root initiation. It is possible, in a hypocotyl, to locate the cell type(s) in which polar auxin transport occurs in the intact plant using microautoradiography. It should also be possible to identify lateral migration of IAA away from the cells involved in polar IAA transport in an intact stem/hypocotyl. Following cutting excision, any changes in the lateral diffusion and redistribution of IAA between different cell types could also be monitored, and the actual location of the cells determined. By monitoring lateral diffusion and redistribution of IAA between different cell types and in cells of different position within the pericycle, a clearer understanding of why only certain cells respond should emerge. Jones (1990) has examined the problem of polar auxin transport in 3.5 day old *Z. mays* seedling shoots using a newly developed technique involving photoaffinity labelling. The data is relevant to auxin and root initiation, because in *Z. mays* it shows that auxin is available to all cells, although at different concentrations, and that internal pathways for auxin movement are also possible and significant. However, the study by Jones (1990) was carried out in excised mesocotyl segments over 4 hours, and it is not clear whether the same distribution of [$^3$H], 5-$N_3$-IAA would have been found in an intact seedling.

By developing immunocytochemical and photoaffinity labelling techniques it could be possible to get a much clearer idea of the role of IAA in the rooting process and in particular, to get more detail about the events within the rooting zone especially within the pericycle and surrounding cells.

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# Appendix.

## A.1 The effect of media on *in vitro* seedling growth.

Aim: an initial experiment to select media in which *in vitro Eucalyptus globulus* seedlings grow. Media were selected based on data from Advanced Technologies (Cambridge) Ltd.

Materials and methods.

A combination of three MS concentrations (full, 1/2, and 1/4 strength), 2 sucrose concentrations (0.03 M and 0.09 M), presence or absence of 0.5% w/v activated charcoal, plus liquid or solid media were tested. Media were made from stock chemicals to avoid the possibility of variation between packets in the commercially prepared MS powder. There were 24 treatments, 8 replicate magentas per treatment and 4 seedlings per magenta. The length of the main root was measured, plus the degree of branching and hairiness was given a mark of one to 5. For the shoot growth leaf number and a mark of 1 to 5 was given for the vigour of the material. The degree of callusing was also estimated.

Results.

Due to contamination only complete 3 replicate magentas were left before any results were taken. Results were taken after 1, 2, and 5 months.

The following observations were made:

(i) Root length, as measured by length of the main root, was better with low salts (Table A.1).

(ii) Shoot growth was better with solid media

(iii) Root growth, (length, degree of branching and hairiness), was vastly superior when grown in liquid media verses solid media. However, shoot callusing and poor shoot growth, which may have been due in part to wetting of the young shoot growth was very apparent when the seedlings were grown in liquid media. It would be

interesting to record the shoot growth with liquid media if there was a simple way of suspending the shoot system above the media.

(iv) The overall optimum, (for shoot and root growth), was 1/2 strength MS, 0.03 M sucrose solidified with 0.7% w/v agar. The benefits of AC was more apparent as the plants aged, this may be due to AC mopping up substances which build up in the media as the plants age, media without AC increased in browning as the plants aged. After about 1 month signs of nutrient deficiency could be seen, plants on media with 0.09 M sucrose were not as affected as those with 0.03 M sucrose.

**Table A.1 Length of the main root (cm) after one month.**

| | Strength of MS media | | |
|---|---|---|---|
| | Quater | Half | Full |
| **Media type.** | X (S.E) | X (S.E) | X (S.E) |
| 0.03M S A - | 4.67 +/- 0.17 | 3.33 +/- 0.29 | 3.67 +/- 0.11 |
| 0.09M S A - | 2.25 +/- 0.31 | 2.50 +/- 0.37 | 3.00 +/- 0.22 |
| 0.03M S L - | 3.67 +/- 0.60 | 2.42 +/- 0.15 | 2.42 +/- 0.18 |
| 0.09M S L - | 3.58 +/- 0.43 | 1.67 +/- 0.17 | 3.25 +/- 0.07 |
| 0.03M S A + | 4.00 +/- 0.26 | 3.50 +/- 0.14 | 2.75 +/- 0.07 |
| 0.09M S A + | 4.42 +/- 0.11 | 3.97 +/- 0.04 | 3.83 +/- 0.05 |
| 0.03M S L + | 3.83 +/- 0.29 | 4.75 +/- 0.07 | 2.92 +/- 0.21 |
| 0.09M S L + | 5.00 +/- 0.06 | 4.33 +/- 0.18 | 3.42 +/- 0.33 |
| Overall X | 3.93 +/- 0.28 | 3.31 +/- 0.18 | 3.16 +/- 0.15 |

Key: S= sucrose; A= solidified with agar (0.7% w/v); L= liquid media (no agar); +/- = with, or, without activated charcoal respectively.

## A.2 *In vivo* rooting of *E. globulus* seedling cuttings.

Aim: To select a medium which produces a high rooting percentage without the curvature of the hypocotyl base (Experiment 4.1a,b).

Materials and methods.

Three media were tested, vermiculite, sand, and a 50:50 mixture of vermiculite and sand. The sand was a 50:50 mixture of fine and course grade, the vermiculite was medium grade. Seedlings with 4 node pairs, ( the second true leaf pair expanding and the third true leaf pair upright), were severed either 1cm below the cotyledons (hypocotyl cuttings), or, 1cm below the first true leaf pair (stem cuttings). There were 3 replicate randomised blocks, with 20 cuttings per cutting type in each block.

Results.

There was no significant difference between the three rooting media tested, but there was a significant difference in the rooting ability of the two cutting types (Table A2). The rooting ability of the hypocotyl cuttings was significantly higher than that of the stem cuttings.

Table A.2 The effect of the rooting media and cutting type on the rooting of *in vivo E. globulus* seedling cuttings.

| | Cutting type | |
|---|---|---|
| Media | Hypocotyl | Stem |
| Vermiculite | 83% | 60% |
| Sand | 87% | 57% |
| Vermiculite and sand | 87% | 53% |

## A.3 The effect of activated charcoal in the rooting medium.

Aim: to determine whether the use of activated charcoal (AC), as used by Advanced Technologies (Cambridge) Ltd., in the second phase of the rooting medium is necessary for a high, synchronous rooting ability. The inclusion of AC in root elongation media is to mop up IBA. High auxin in the later stages of adventitious root formation has been reported as being inhibitory (Thiman, 1936; Liu and Reed, 1992). One problem in having AC evenly distributed throughout the media when ARF is being studied is that obscures the view, so the time of root emergence and determining the position from the cutting base as the roots emerge is impossible. If AC was effective when located on the base of the magenta it would overcome the problem of AC blocking the visibility of root emergence.

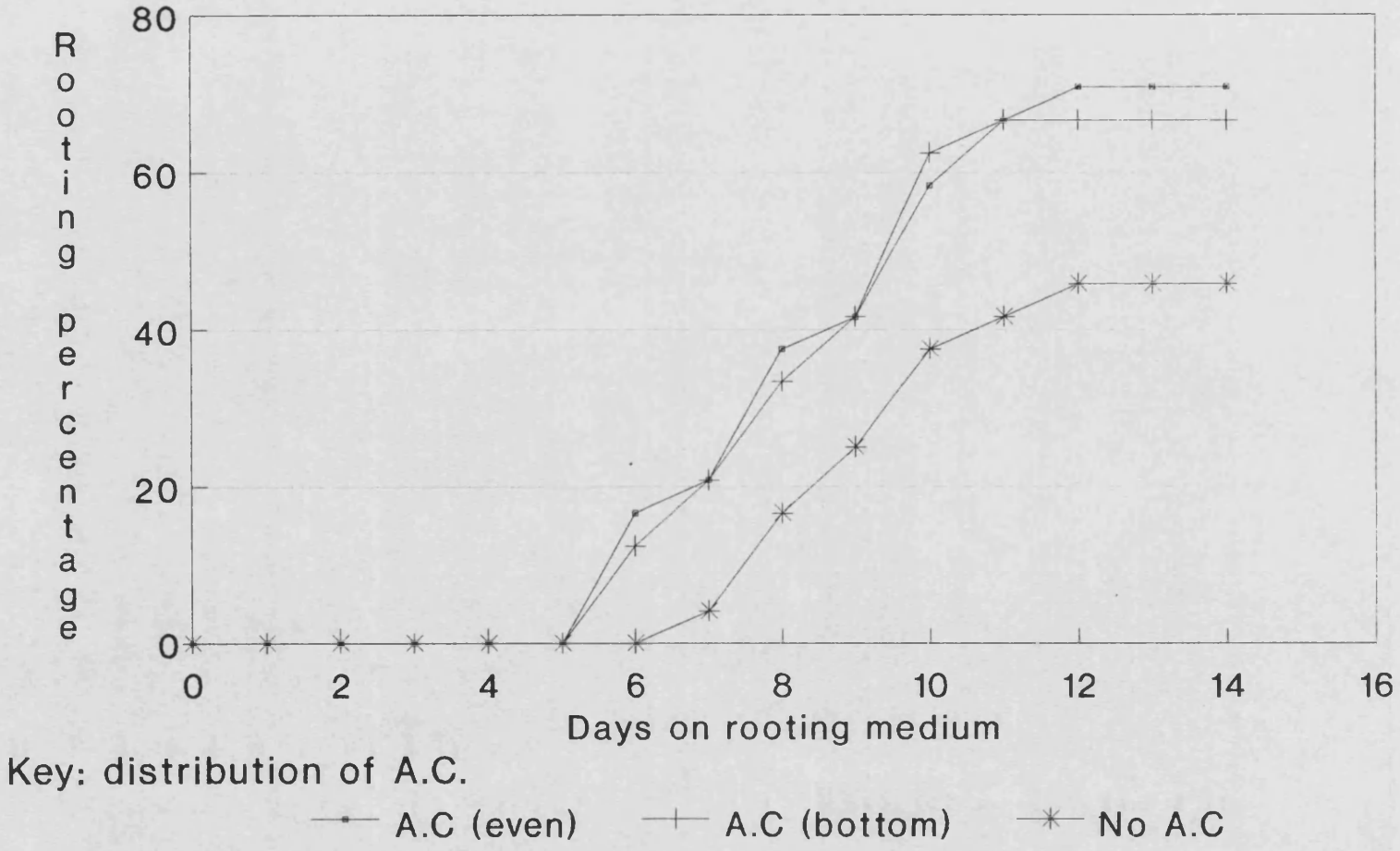
Materials and methods.

. There were 6 replicate magentas each containing 4 explants, the stock material was 3.5 months old and explants consisted of the apical 3 node pairs with the basal leaves removed. For five days all explants were placed on a 10 μM IBA pulse, root initiation medium All three treatments, (zero AC, 0.3% w/v AC evenly distributed, and 0.3% w/v AC allowed to settle near on the bottom of the magentas), had 1/4 strength MS, 0.03 M sucrose, and were solidified with 0.15% w/v phytagel.

Results.

The distribution of activated charcoal did not make a significant difference on the rooting ability of the explants (Figure A.1). However, when AC was omitted the rooting ability dropped from around 70% to just over 40%. In addition the general vigour of the roots appeared poorer without AC. Being able to let the AC settle on the base of the magentas will allow the time of root emergence to be monitored without the need of removing the explants.

**Figure A.1 The effect of distribution of activated charcoal (A.C.) on the *in vitro* rooting of *E. globulus* explants.**




**Rooting chambers = magentas**

## A.4 The effect of feeding glasshouse-grown *E. globulus* seedlings on the subsequent rooting of hypocotyl cuttings from the material.

Aim:

To see whether nutrients could be limiting the rooting ability of glasshouse-grown *E. globulus* seedling cuttings.

Materials and methods.

Seedlings were grown in Fisons F1 compost and fed at 10 days after germination. Peters M-77 general purpose, water soluble compost (N-P-K, 20-20-20), at either 0, 0.05% w/v, or 0.1% w/v was used. Cuttings, severed 1cm beneath the cotyledons, were struck in vermiculite at the following ages, 12, 16, 20, and 24 days old and kept at 25°C, 60% relative humidity, with continuous lighting. There were 3 replicates per treatment, with 16 cuttings per replicate.

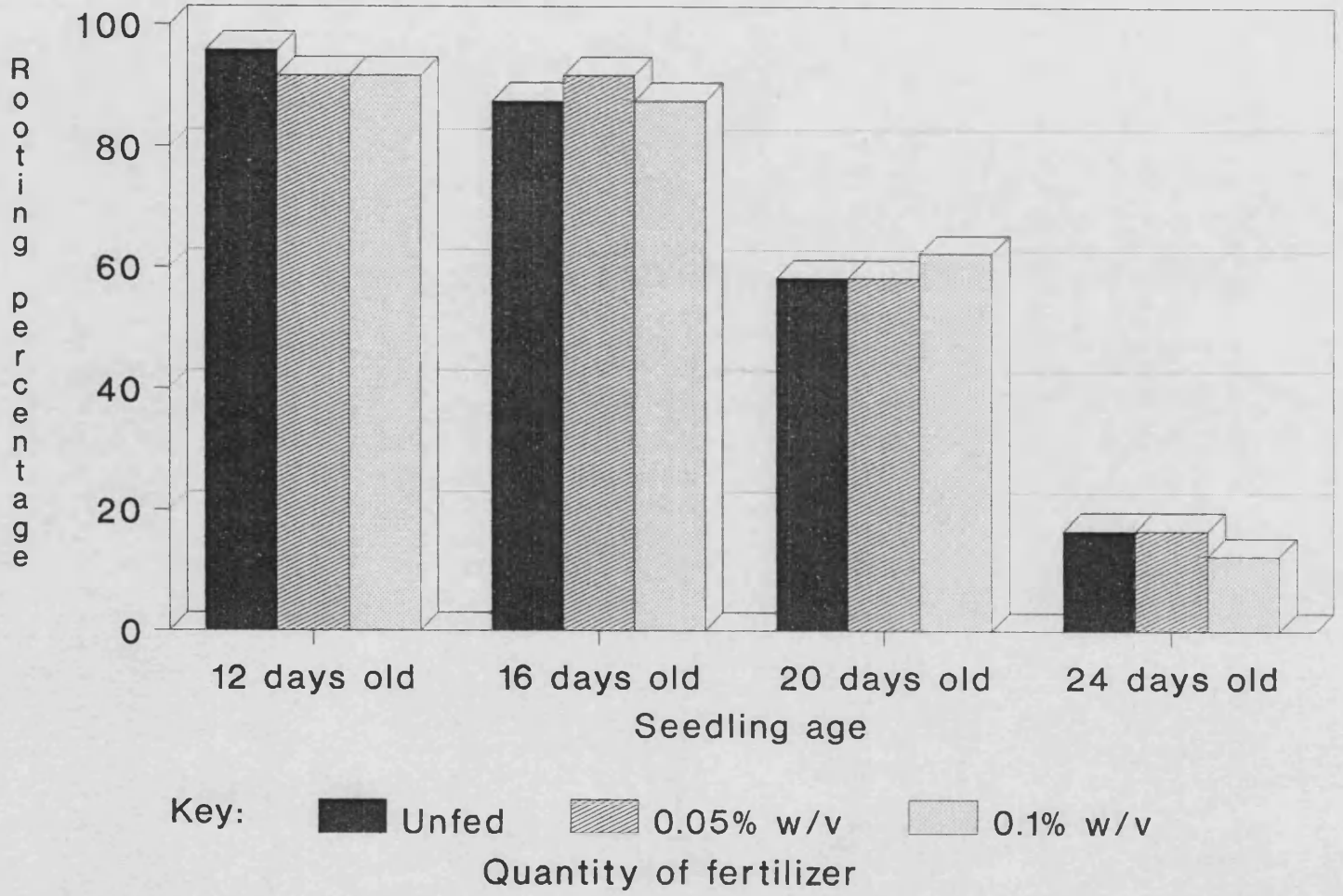
Results.

There was no significant effect of application of the three fertiliser concentrations used on the subsequent rooting ability of the hypocotyls tested (Figure A.2). Plants are routinely fed every couple of weeks and when the plants are grown for more than 3 weeks, 0.3% w/v general purpose fertiliser granules are incorporated in the compost, so a lack of nutrients should not be a limiting factor in the rooting trials.

## A.5 Preparation of Sephadex QAE-25.

Sephadex QAE-25 was placed in a conical flask, covered in a 1M solution of sodium formate, shaken and then left to settle. After approximately 4 hours, when the suspension had settled, the fine particles which float near the surface of the liquid were carefully poured-off , the 1M. sodium formate topped-up and then the flask was again shaken and left to settle; this occurred about 4 times to eliminate the fine particles.

**Figure A.2 The effect of feeding *E. globulus* seedlings with a general purpose fertilizer on the subsequent rooting ability of cuttings.**



When the fine particles had been removed the Sephadex was washed twice in distilled water before being stored in 1% formic acid.

## A. 6 Preparation of Haupt's solution.

Gelatin 1g.

Phenol crystals 2g.

Glycerol 15 ml.

Procedure: Disolve the gelatin in 100ml of distilled water at 60°C, add the phenol and glycerol, stir and filter.

## A.7 TBA dilution series.

| Water | Ethanol (95%) | TBA | Ethanol (100%) |
|---|---|---|---|
| 50 | 40 | 10 | - |
| 30 | 50 | 20 | - |
| 15 | 50 | 35 | - |
| - | 50 | 50 | - |
| - | - | 75 | 25 |

Key: figures = percentages.